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Metabolismo de galotaninos en bacterias con actividad tanasa presentes en el tracto gastrointestinal humano.

Memoria presentada por

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CERTIFICAN:

Que la licenciada **Natalia Jiménez Martín** ha realizado bajo su dirección, en el Departamento de Procesos del Instituto de Ciencia y Tecnología de los Alimentos y Nutrición, el trabajo de investigación titulado **"Metabolismo de galotaninos en bacterias con actividad tanasa presentes en el tracto gastrointestinal humano"** que presenta en el Departamento de Química-Física Aplicada de la Facultad de Ciencias de la Universidad Autónoma de Madrid para optar al grado de Doctora.

Y, para que así conste a los efectos oportunos, firman el presente certificado en Madrid a 10 junio de 2014.

Directoras de la tesis

Dra. Rosario Muñoz Dra. Blanca de Las Rivas

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Introducción

Lista de abreviaturas

aa: Aminoácidos
Ala: Alanina
Arg: Arginina
Asp: Aspártico
ATCC: *American Type Culture Collection*
BAL: Bacteria lácticas
CECT: Colección Española de Cultivos Tipo
Da: Daltons
DEAE: Dietilaminoetanol
DSM: Colección Alemana de microorganismos
DSMO: Dimetilsulfóxido
EDTA: Ácido etilen-diamino-tetra-acético
g: Gramos
Gln: Glutamina
Glu: Glutámico
Gly: Glicina
GRAS: Reconocidos generalmente como seguros
h: horas
His: Histidina
HOHN: 3-hidroxi-5-oxohexanoato
HPLC: Cromatografía de líquidos de alta eficacia
Kb: Kilobase
kDa: Kilodalton
L: Litro
Lys: Lisina
Mb: Megabase
mg: miligramos
min: minuto
mM: milimolar
Ser : Serina
Sp.: Especie
Subsp.: Subespecie
t max: Tiempo máximo
Tª: Temperatura
TGI: Tracto gastrointestinal
U: Unidades

Introducción

1. COMPUESTOS FENÓLICOS

1.1. Definición y clasificación

Bajo el nombre de compuestos fenólicos se engloba un numeroso y heterogéneo conjunto de moléculas que poseen en su estructura uno o varios anillos aromáticos sustituidos por uno o varios grupos hidroxilo (Croteau *et al*., 2000). Los compuestos fenólicos constituyen el principal grupo de metabolitos secundarios en el reino vegetal y generalmente están involucrados en la protección contra la radiación ultravioleta o contra patógenos (Manach *et al*., 2004). Estos compuestos representan un ejemplo de la plasticidad del metabolismo de las plantas que les permite adaptarse a cambios medioambientales tanto bióticos como abióticos (Boudet, 2007). Los compuestos fenólicos funcionan, por ejemplo, como señales para el establecimiento de relaciones simbióticas con rizobios o atrayentes para la polinización, también como aislantes que impermeabilizan las paredes celulares frente a gas y agua y como materiales estructurales que aportan estabilidad a las plantas. Los compuestos fenólicos se localizan principalmente en frutos u órganos aéreos jóvenes, por lo que se consumen diariamente en la dieta (Harborne & Williams, 2000).

La mayoría de los compuestos fenólicos se sintetizan vía fenilalanina cuyo compuesto central es el fenilpropanoide. A partir de ahí derivan diversas ramas metabólicas que originan los precursores para la síntesis de monoligninas, flavonoides, estilbenos, ésteres fenólicos, etc. (Boudet, 2007). Las estructuras químicas de los compuestos fenólicos son muy diversas, desde ácidos fenólicos simples de bajo peso molecular hasta moléculas poliméricas de elevada masa molecular como los taninos condensados y los hidrolizables. Los compuestos fenólicos pueden clasificarse en función del número de anillos fenólicos que contienen y de los elementos estructurales que unen a los anillos entre sí. Basándose en estas características se pueden distinguir: ácidos fenólicos (derivados del ácido benzoico o del ácido cinámico), flavonoides (flavonoles, flavonas, isoflavonas, flavanonas, antocianidinas y flavanoles), estilbenos, lignanos y taninos (Figura 1) (Manach *et al*., 2004).

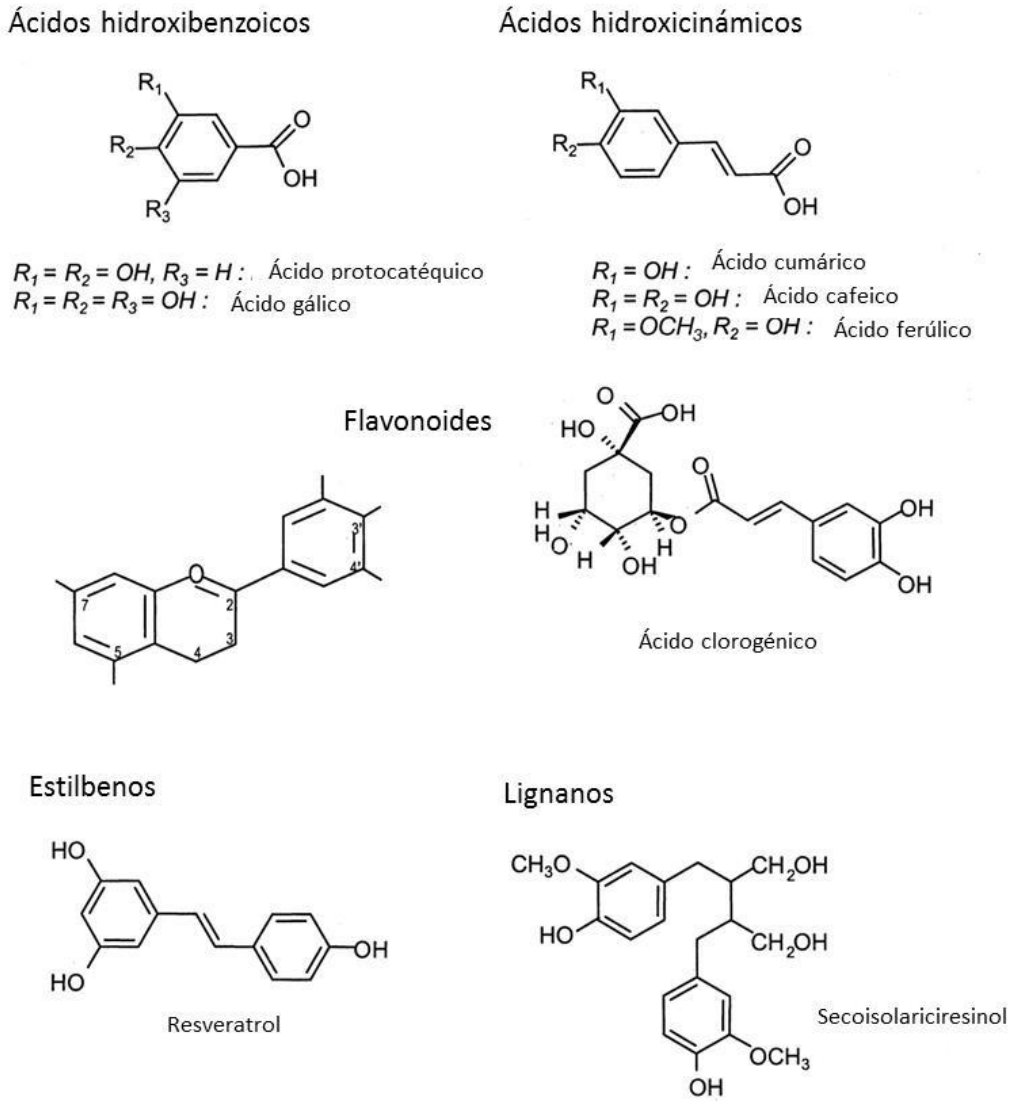

Figura 1. Clasificación de los compuestos fenólicos en función del número de anillos fenólicos y de los elementos estructurales que unen a los anillos entre sí (Manach *et al*., 2004).

Los ácidos fenólicos son fenoles que poseen un ácido carboxílico como grupo funcional. Los ácidos fenólicos se consideran el grupo de compuestos fenólicos mayoritario presente en la dieta. Este grupo se puede dividir a su vez en ácidos hidroxibenzoicos, componentes de estructuras complejas como los taninos hidrolizables (Manach *et al*., 2004) y ácidos hidroxicinámicos, como los ácidos *p*-cumárico, cafeico y ferúlico (Haminiuk *et al*., 2012).

Los flavonoides son compuestos fenólicos de gran variabilidad estructural. Se encuentran ampliamente distribuidos en las frutas y son antioxidantes naturales (Haminiuk *et al*., 2012). Los flavonoides son otro grupo de compuestos fenólicos abundante en la dieta

(Robbins, 2003) que se presentan generalmente como glucósidos y ésteres (Haminiuk *et al*., 2012).

Los estilbenos se encuentran en bajas cantidades en la dieta. Su presencia se restringe básicamente a uva, cacahuetes y distintas bayas del género *Vaccinium* (Gürbüz *et al*., 2007). En la actualidad, uno de los estilbenos más importante es el resveratrol. Numerosos estudios indican que el resveratrol previene el cáncer y enfermedades coronarias, neurológicas y degenerativas (Haminiuk *et al*., 2012).

Los lignanos están formados por unidades de 2-fenilpropano (Manach *et al*., 2004). Se encuentran en una gran variedad de plantas que incluyen las semillas de lino, semillas de calabaza, semillas de ajonjolí, centeno, soja, brócoli, frijoles, y algunas bayas. Aunque están ampliamente distribuidos en dichas semillas, se encuentran en baja cantidad (Crosby, 2005).

Los taninos están presentes en multitud de alimentos como fresas, moras, plátanos, nueces y en bebidas como vino y té. Además se encuentran en todas las partes de las plantas como raíces, corteza, ramas, semillas y hojas. El término tanino se utilizó por primera vez en 1796, como consecuencia del uso de estos compuestos en las curtidurías. Sin embargo este grupo de compuestos fenólicos fue definido por primera vez por Bate Smith y Swain en 1962 como compuestos fenólicos solubles en agua con pesos moleculares comprendidos entre 500 y 3000 Da. Actualmente se conoce que algunos no son solubles en agua y que sus pesos varían entre 500 y 30000 Da (Serrano *et al.*, 2009). Los taninos se dividen en dos grupos, taninos condensados y taninos hidrolizables. Los taninos condensados están formados por la polimerización de flavonoides (Young *et al*., 1985). Los taninos hidrolizables se pueden diferenciar en galotaninos, cuya hidrólisis libera ácido gálico, y elagitaninos que liberan ácido elágico tras su hidrólisis (Figura 2) (Serrano *et al*., 2009). Los taninos hidrolizables están presentes en frutas, verduras y otros alimentos. Destacando su presencia en plantas pertenecientes al orden Rosidae (fresas, frambuesas, moras, granadas, mango, nueces etc.) así como en los órdenes Dilleniidae y Hamamelidae pero en menor proporción (Arapitsas, 2012).

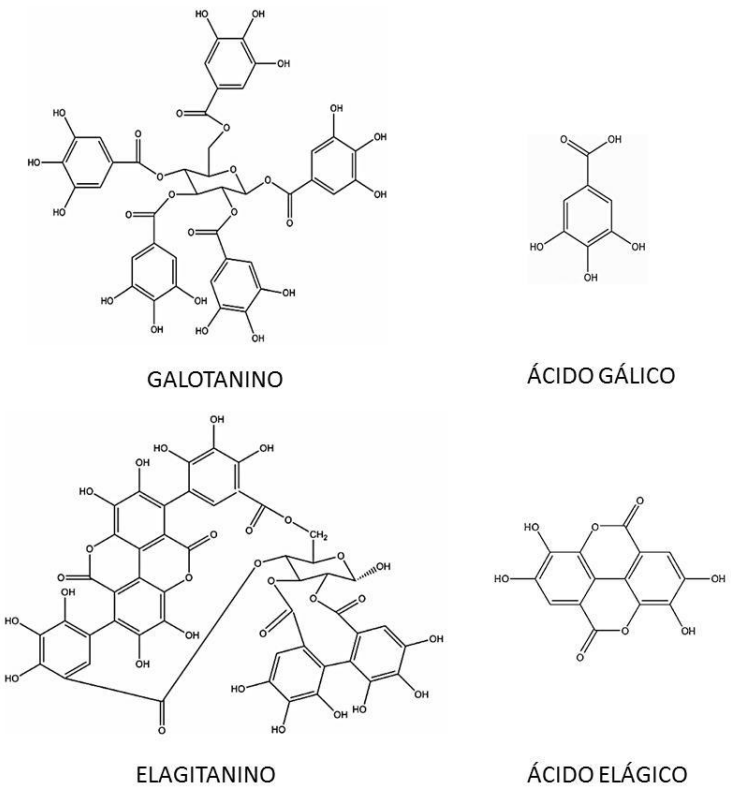

Figura 2. Estructura de taninos hidrolizables y de sus ácidos estructurales. Modificada de Serrano *et al*., (2009).

En los últimos años se ha producido un creciente interés por estos compuestos debido a sus propiedades antioxidantes, a su gran abundancia en la dieta y a su posible papel en la prevención de enfermedades como cáncer, alteraciones cardiovasculares y problemas degenerativos (Manach *et al*., 2004).

1.2. Influencia de los compuestos fenólicos en los alimentos

Los compuestos fenólicos son responsables de algunas de las características organolépticas de los alimentos de origen vegetal, como el color de las frutas (Cheynier, 2012). Los flavonoides aportan colores amarillos, rojos y azules a la fruta (Lampila *et al*., 2009). Las antocianinas en medios ácidos (pH ≤2) aportan coloración roja, sin embargo a pH más altos, como consecuencia de un proceso de hidratación y equilibrio ácido-base, esta coloración se convierte en incolora o azulada, respectivamente (Daayf *et al.,* 2012). Los compuestos fenólicos también pueden producir efectos sensoriales no deseados, como por ejemplo el

pardeamiento de los alimentos, ocasionado por la oxidación de los derivados de los ácidos hidroxicinámicos por acción de las enzimas oxidasas presentes en los tejidos vegetales.

La oxidación de los compuestos fenólicos, catalizada por polifenoloxidasas, lacasas y peroxidasas, también contribuye al sabor y a la textura de los alimentos (Daayf *et al.,* 2012). La astringencia es una característica que presentan algunas bebidas como vino, sidra, té, etc. y se identifica como una pérdida de lubricación y sequedad en el paladar y está asociada a la interacción de los compuestos fenólicos con las proteínas de la saliva ricas en prolina (Haslam, 2007). Los compuestos fenólicos también contribuyen al amargor de los alimentos, atribuido principalmente a la presencia de flavonoides.

La contribución de los compuestos fenólicos en el aroma se debe esencialmente a la presencia de fenoles volátiles. Los fenoles volátiles pueden aparecer por hidrólisis de alcoholes superiores o por el metabolismo de microorganismos, como levaduras y bacterias lácticas (Rodríguez *et al*., 2009). Por ejemplo, en el vino la descarboxilación de los ácidos *p*-cumárico y ferúlico origina 4-vinil fenol y 4-vinil guayacol que son compuestos aromáticos e intermediarios útiles en la producción biotecnológica de nuevos aromas. Sin embargo la posterior reducción de los vinil fenoles origina etil fenol y etil guayacol, que se consideran aromas desagradables y son responsables de alteraciones en las propiedades organolépticas de algunos alimentos (Chatonnet *et al*., 1992).

La concentración de compuestos fenólicos en los alimentos depende de factores genéticos, medioambientales y tecnológicos algunos de los cuales se pueden controlar para optimizar el contenido de estos compuestos en los alimentos (Manach *et al*., 2004).

1.3. Compuestos fenólicos y salud del consumidor

En general se considera que los compuestos fenólicos ejercen efectos beneficiosos en la salud del consumidor. Numerosos estudios científicos y evidencias epidemiológicas han asociado el consumo de verdura y fruta con una reducción del riesgo de cáncer y enfermedades cardiovasculares y degenerativas (Arapitsas, 2012). Además se les atribuye propiedades antioxidantes, antimicrobianas y antivirales, antimutagénicas o un efecto cardioprotector (Serrano *et al*., 2009). Sin embargo su consumo también se ha asociado con cáncer y hepatotoxicidad y se ha descrito que poseen actividad antinutritiva. Estas

propiedades antagónicas son destacables en el caso de los taninos, compuestos considerados como "una espada de doble filo" (Chung *et al*., 1998).

Se ha descrito que los taninos poseen actividad antioxidante, antimutagénica y anticancerígena. Los estudios realizados demuestran que la ingesta de zumo de arándanos, pasas o vino incrementa la capacidad antioxidante en plasma y reduce la oxidación lipídica, lo que previene de enfermedades cardiovasculares (Heinonen, 2007). Además se ha constatado la actividad antimicrobiana de numerosos taninos frente a un gran número de especies de hongos, bacterias y sobre algunos virus (Chung *et al*., 1998). Se ha demostrado la inhibición de los efectos citopáticos del VIH y la expresión del antígeno VIH en células MT-4 por taninos (Haslam, 1996).

Por otro lado, sin embargo también se ha descrito que los taninos poseen propiedades nutricionalmente indeseables. En animales cuyas dietas son ricas en taninos se produce una pérdida de peso y una reducción de su crecimiento. Los taninos, son capaces de formar complejos con proteínas, lo que puede ser causa de inhibición enzimática y de ese modo interferir en la digestión y absorción de nutrientes (Serrano *et al*., 2009). Además, los taninos también forman complejos con iones metálicos como hierro, manganeso, aluminio, calcio, etc. Se ha descrito que los taninos forman complejos con el hierro divalente que ocasiona una reducción en su absorción, y también reducen la utilización de vitaminas y minerales (Chung *et al*., 1998). Las proteínas de la saliva, ricas en prolina, poseen alta afinidad por los taninos. La unión entre estas proteínas y los taninos es la responsable de la astringencia de los alimentos y de las bebidas ricas en taninos.

La influencia de los compuestos fenólicos en la salud depende de la cantidad consumida y de su biodisponibilidad. La biodisponibilidad hace referencia a la fracción de compuesto fenólico que llega hasta el sistema circulatorio (Landete, 2012). En relación a su biodisponibilidad, la mayoría de los compuestos fenólicos se encuentran en los alimentos en forma de ésteres, glucósidos o polímeros que no pueden absorberse. Estos compuestos para absorberse deben ser hidrolizados mediante enzimas intestinales o mediante la actividad enzimática presente en la microbiota intestinal (Manach *et al*., 2004). La influencia de los compuestos fenólicos en el consumidor va a depender, por tanto, de las variaciones interindividuales en la composición de la microbiota del tracto gastrointestinal (TGI) que afectan a la biotransformación de estos compuestos (Chen *et al*., 2012).

En los animales herbívoros la microbiota del rumen posee bacterias capaces de degradar taninos, lo que reduce su actividad antinutritiva (Goel *et al*., 2005). Además de la microbiota del rumen, se ha descrito que otros microorganismos presentes en el TGI son capaces de degradar los complejos formados entre las proteínas y los taninos. Osawa (1990) describió por primera vez que especies del género *Streptococcus* aisladas de heces de koalas eran capaces de degradar *in vitro* complejos formados por proteínas y taninos (ácido tánico). Posteriormente, se describió que estos complejos proteína-ácido tánico también se degradaban por *Pantoea agglomerans* (Osawa *et al*., 1992) y *Lonepinella koalarum*, una bacteria aislada de heces de koala (Osawa *et al*., 1995). También se han aislado bacterias capaces de degradar taninos a partir de heces de humanos o de alimentos fermentados (*Lactobacillus plantarum*, *Lactobacillus paraplantarum* y *Lactobacillus pentosus*) (Osawa *et al*., 2000). La presencia en el TGI humano de bacterias capaces de degradar los complejos formados entre proteínas y taninos hace que estas bacterias puedan modular los efectos producidos por los taninos de la dieta.

2. MICROBIOTA DEL TRACTO GASTROINTESTINAL

2.1. Definición y clasificación

La microbiota es el conjunto de microorganismos que coexisten en relación simbiótica con el hospedador. La microbiota que coloniza el TGI humano es de gran complejidad. El conjunto de bacterias que constituyen esta microbiota supone 2 kg de nuestro peso (Tomás-Barberán *&* Mine, 2013), la distribución de los microorganismos no es homogénea a lo largo del TGI y se estima que el colon contiene el 70% de estos microorganismos. Los valores varían desde 10^1 a 10^3 bacterias por gramo en el estómago y duodeno, de 10^4 a 10^7 en yeyuno e íleon, hasta 10^{11} a 10^{12} bacterias por gramo en el colon (Sekirov *et al*., 2010). La microbiota del TGI humano está compuesta mayoritariamente por bacterias de los filos *Bacteriodetes* y *Firmicutes* y en menor proporción de los filos *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Fusobacteria* y *Cyanobacteria* (Sekirov *et al*., 2010). Además de la distribución no homogénea de los microorganismos, existe una alta variación interindividual de las especies que constituyen la microbiota (He *et al*., 2013; Sekirov *et al*., 2010) (Figura 3).

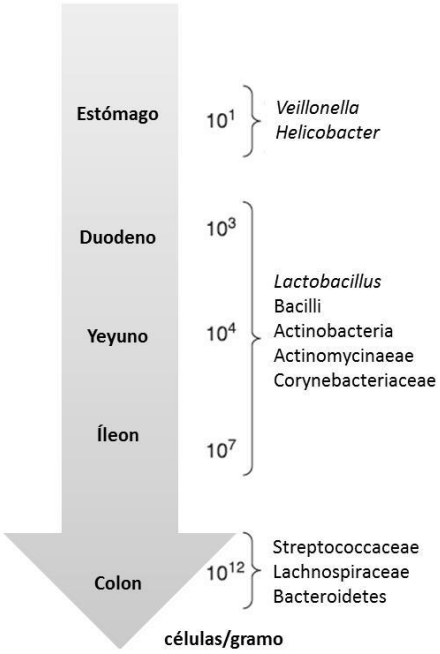

Figura 3 Composición de la microbiota del TGI. Modificada de Sekirov *et al*., (2010).

2.2. Dieta, microbiota y salud

La microbiota influye en la colonización del TGI por parte de otros microorganismos y en la integridad de la barrera epitelial, lo que favorece la protección contra patógenos mediante la creación de condiciones adversas para su crecimiento y por exclusión competitiva (Kleerebezem & Vaughan, 2009; Nyangale *et al*., 2012). Por ello la microbiota intestinal tiene una función imprescindible en el desarrollo del sistema inmune. Además se ha descrito una asociación entre ciertas patologías y diferencias en la composición de la microbiota (Huertas & Michán, 2014). La dieta es uno de los principales factores que modifican la composición de la microbiota. Algunas bacterias metabolizan determinados componentes de los alimentos para obtener energía, por lo que dichas bacterias pueden crecen mejor que las que no pueden metabolizar dichos compuestos. Por ejemplo, se ha observado que las dietas libres de gluten están relacionadas con una reducción de poblaciones bacterianas consideradas saludables, como los géneros *Bifidobacterium* y *Lactobacillus*, y un aumento de patógenos oportunistas (He *et al*., 2013).

Se ha descrito que la microbiota del TGI puede adaptar su metabolismo a las condiciones variables del intestino en respuesta a la disponibilidad de substratos. Además la

microbiota se adapta a la presencia de otros microorganismos, con el objeto de maximizar su supervivencia, mediante la utilización de nutrientes de forma selectiva (Sekirov *et al*., 2010). Los factores que afectan a la composición de la microbiota son numerosos, pueden ser factores genéticos, geográficos, modo de nacimiento, dieta, uso de antibióticos o uso de probióticos y prebióticos (Nyangale *et al*., 2012). Todo ello influye y determina una evolución conjunta entre los diferentes microorganismos y el hospedador. Parte de esta coevolución implica la transferencia horizontal de genes entre los microorganismos de la microbiota para ganar funciones y adaptarse a nuevas condiciones ambientales (He *et al*., 2013).

2.3. Compuestos fenólicos y microbiota

La biodisponibilidad de los compuestos fenólicos, como se ha comentado anteriormente, se define como la fracción de compuesto fenólico que llega hasta el sistema circulatorio (Landete, 2013). Su absorción depende de su estructura química primaria, que a su vez depende de factores como el grado de glicosidación, acilación, de su estructura básica, de su conjugación con otros fenoles, del grado de polimerización y de su solubilidad (Landete, 2013). La mayoría de los compuestos fenólicos se presentan en forma de ésteres, glicósidos o polímeros que no pueden absorberse, por lo que deben ser hidrolizados por las enzimas intestinales o por la microbiota (Manach *et al*., 2004). Durante la digestión se absorben parcialmente en el intestino delgado. Durante su absorción los polifenoles se conjugan en el intestino delgado y posteriormente en el hígado. Este proceso sirve para destoxificar y facilitar su eliminación mediante la bilis o la orina (Manach *et al*., 2004). Posteriormente, los compuestos fenólicos no absorbidos y la fracción re-excretada por la bilis, llegan al colon donde se degradan por acción de la microbiota hasta ácidos fenólicos simples. Finalmente, los microorganismos del colon metabolizan estos fenoles simples hasta compuestos no aromáticos (Moco *et al*., 2012) (Figura 4).

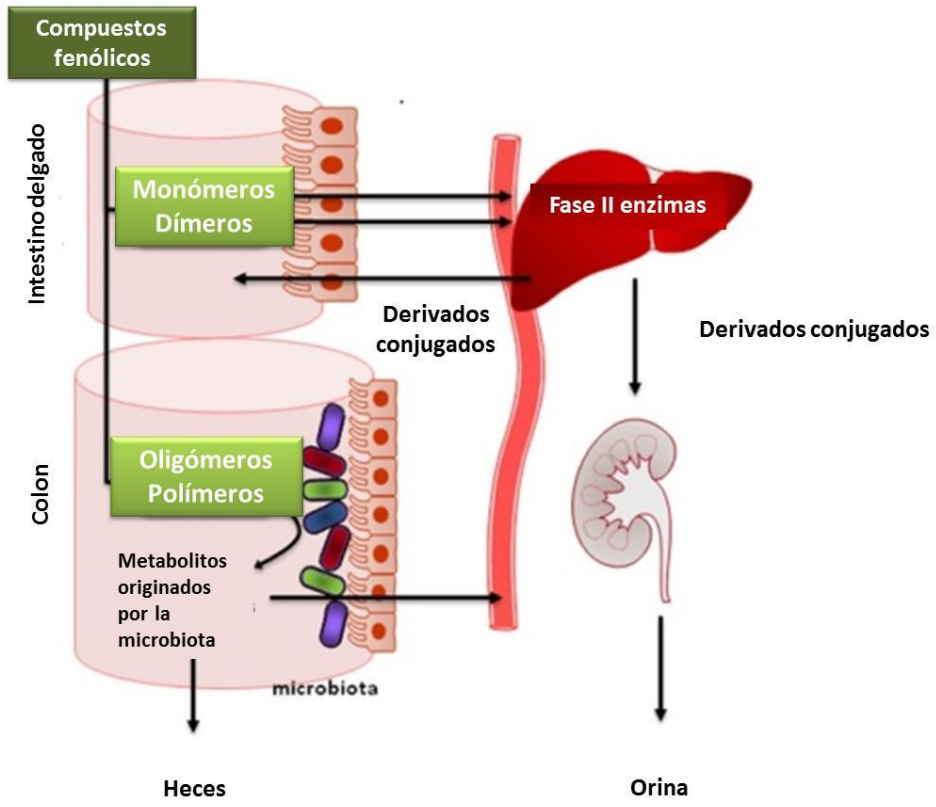

Figura 4. Ruta metábolica de los compuestos fenólicos en el TGI humano. Modificada de Pérez-Cano *et al*. (2013).

Los taninos pueden ser beneficiosos o perjudiciales para la salud dependiendo de su concentración y de su estructura (Goel *et al*., 2005). La microbiota intestinal puede influir también en el efecto de los taninos en la salud del consumidor. Se observó que en la orina de los consumidores existía una gran variabilidad de metabolitos derivados de la degradación de los polifenoles del café y del cacao. Esa variabilidad reflejaba los diferentes metabolismos utilizados por las diferentes microbiotas presentes en el colon de cada consumidor (Moco *et al*., 2012). La dieta también afecta a la composición de la micriobiota y por lo tanto influye en la salud del consumidor. Se ha descrito que los compuestos fenólicos del cacao y del café modulan la microbiota intestinal, aumentan el número de bacterias de las especies *Clostridium coccoides*, *Eubacterium rectale*, *Escherichia coli* y bacterias del género *Bifidobacterium* mientras que reducen el crecimiento de *Clostridium histolyticum*. Sin embargo, la degradación de los compuestos fenólicos presentes en las fracciones insolubles del cacao se relaciona con un aumento del número de bacterias de los géneros *Lactobacillus* y *Bifidobacterium*. A su vez este aumento se asocia con una reducción significativa de los niveles de triglicéridos en plasma, lo que sugiere un efecto beneficioso en la salud (Moco *et al*., 2012). Se ha descrito que

al suministrar extracto de té verde suplementado con *L. plantarum* DSM 15313 durante 22 semanas se aumenta el número de bacterias del grupo de *Lactobacillus* y se reduce la inflamación intestinal originada por una dieta rica en grasa (Axling *et al*., 2012). Estudios *in vitro* indican que los elagitaninos son capaces de inhibir el crecimiento de las especies del género *Clostridium* y reducir el crecimiento de la especie patógena *Staphylococcus aureus* mientras que afectan mínimamente al crecimiento de cepas probióticas de especies del género *Lactobacillus* y *Bifidobacterium* (Etxebarria *et al*., 2013). También se ha descrito que los compuestos fenólicos y sus metabolitos pueden afectar a la ecología del TGI modificando la microbiota mediante mecanismos bacteriostáticos o bactericidas (Etxebarria *et al*., 2013).

Por todo ello, tiene una gran relevancia el estudio de cómo la microbiota del TGI metaboliza los compuestos fenólicos presentes en los alimentos, así como el análisis de los metabolitos que se producen y de las bacterias que lo llevan a cabo. Además resulta interesante conocer cómo los compuestos fenólicos pueden modificar la microbiota del TGI, ocasionando cambios en sus poblaciones que pueden afectar a la salud del consumidor.

3. TANINOS Y BACTERIAS

Se han realizado estudios que indican que el efecto inhibitorio de los taninos en las bacterias del TGI se debe fundamentalmente a la capacidad que tienen los taninos para formar complejos con polímeros y minerales, y a su interacción con la membrana y pared bacteriana y/o con las proteínas extracelulares (Cueva *et al*., 2010). Los microorganismos han desarrollado una serie de mecanismos adaptativos para contrarrestar el efecto de los taninos. Entre ellos se encuentra la secreción de polímeros de unión que limitan sus efectos nocivos o la presencia de enzimas capaces de degradar estos compuestos (Scalbert, 1991). Otro mecanismo adaptativo es la producción de polisacáridos extracelulares, que envuelven la pared celular y la separan de los taninos. Además se ha descrito que hay un aumento de la expresión de los genes que intervienen en la producción de polisacáridos extracelulares en respuesta a la concentración de taninos en *S. gallolyticus* (Goel *et al*., 2005). Por otro lado, las bacterias son capaces de reparar y modificar la membrana para contrarrestar el efecto de los taninos. Se ha descrito que pueden incrementar el número de ácidos grasos insaturados, modificar su isomerización de *trans* a *cis* y cambiar la carga de la cara externa de la membrana (Smith *et al*., 2005). Los taninos pueden unirse a iones metálicos, lo que puede conllevar por ejemplo una reducción de

la disponibilidad de hierro. La producción de enzimas no dependientes de estos metales o la utilización de enzimas alternativas son también mecanismos adaptativos que han desarrollado las bacterias para contrarrestar el efecto de los taninos (Smith *et al*., 2005).

3.1. Metabolismo bacteriano de taninos

A pesar del carácter antimicrobiano de los taninos se han descrito numerosas especies de bacterias capaces de metabolizar estos compuestos. Lewis *&* Starkey (1969) describieron por primera vez una bacteria aerobia, *Achromobacter,* capaz de degradar galotaninos. Deschamps *et al*. (1980) aislaron a partir de ambientes en los que las concentraciones de taninos son elevadas, cepas pertenecientes a los géneros *Bacillus, Staphylococcus* y *Klebsiella* capaces de utilizar ácido tánico como única fuente de carbono. También aislaron bacterias capaces de degradar taninos condensados e hidrolizables. Este mismo grupo describió por primera vez la producción bacteriana de una enzima tanasa extracelular a partir de cultivos de *Bacillus pumilus*, *Bacillus polymyxa*, *Corynebacterium sp*. y *Klebsiella pneumoniae*. Como productos de la degradación del ácido tánico se detectaron ácido gálico y glucosa (Deschamps *et al*., 1983). Además, se han encontrado cepas bacterianas capaces de degradar taninos en el intestino de animales consumidores de forrajes que contienen una alta concentración de taninos. La presencia de estas cepas supondría una ventaja adaptativa para el animal, puesto que su presencia contrarresta el efecto antinutritivo de los taninos (Bernays *et al*., 1989). Entre las bacterias capaces de degradar taninos se encuentran cepas de las bacterias *Streptococcus gallolyticus, Eubacterium oxidoreducens* y *Selenomonas ruminantum* (Krumholz *&* Bryant, 1986; Nelson *et al*., 1995; Nelson *et al*., 1998; O'Donovan *&* Brooker, 2001; Odenyo *et al*., 2001; Odenyo *&* Osuji, 1998). También se han aislado bacterias capaces de degradar taninos en el intestino humano y en alimentos fermentados de origen vegetal (Osawa *et al*., 2000; Vaquero *et al*., 2004).

En los mecanismos de degradación de galotaninos, se han propuesto rutas de degradación aerobias y anaerobias. En una primera etapa común a las rutas aerobias y anaerobias, la enzima tanasa (E.C.3.1.1.20) cataliza la hidrólisis de los enlaces éster presentes en los galotaninos generando ácido gálico y glucosa (Figura 5).

En la ruta aerobia, los monómeros de ácido gálico, generados por acción de la enzima tanasa, se degradan mediante un proceso de rotura oxidativa y se producen ácidos alifáticos, que entran en el ciclo del ácido cítrico (Field *&* Lettinga, 1992). Kumar *et al*. (1999) han

propuesto una ruta alternativa para la degradación del ácido gálico en *Citrobacter freundii*. Estos autores sugieren que, en primer lugar, ocurre la descarboxilación del ácido gálico a pirogalol por una enzima galato descarboxilasa y posteriormente, una dioxigenasa permite la apertura del anillo aromático y la formación de ácido 2-hidroximucónico, el cual, finalmente se transforma en ácido pirúvico y entra en el ciclo de Krebs (Figura 5).

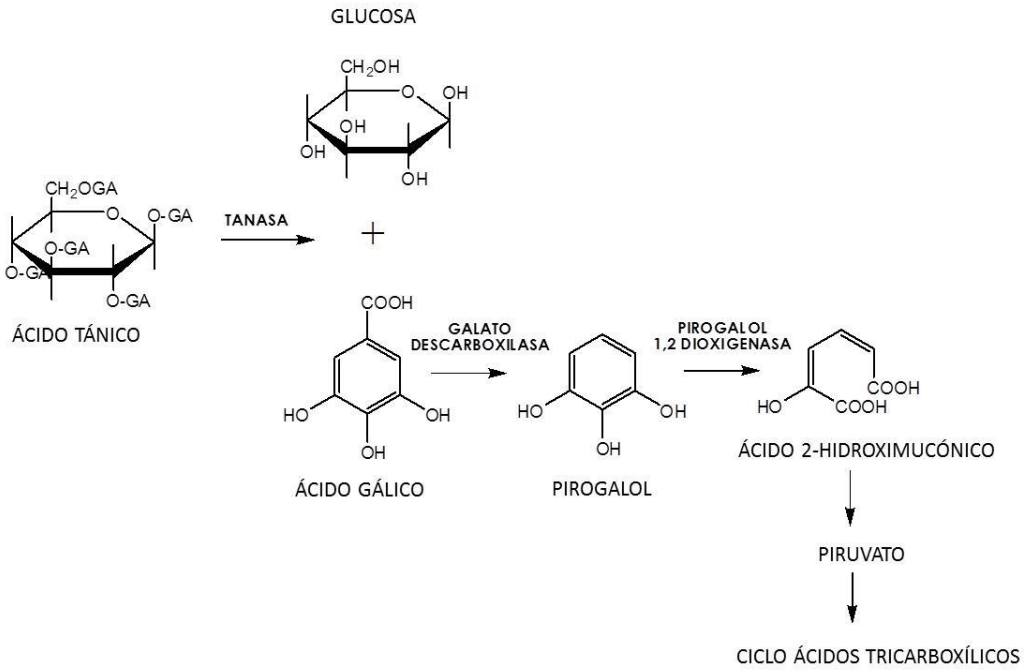

Figura 5. Ruta propuesta para la degradación aerobia de ácido tánico. Modificada de Kumar *et al*. (1999).

En la ruta anaerobia, tras la etapa común, el siguiente paso de la biotransformación de galotaninos es la descarboxilación del ácido gálico para formar pirogalol. En varias especies de bacterias esta etapa de descarboxilación es la etapa final de la biotransformación de galotaninos. Estas bacterias, principalmente aisladas del rumen de animales herbívoros, no tienen capacidad metabólica para la apertura del anillo aromático (Chamkha *et al*., 2002; O'Donovan *&* Brooker, 2001; Odenyo *et al*., 2001). En los casos en los que la etapa de descarboxilación no es la etapa final, el pirogalol se convierte en fluoroglucinol por la acción de una pirogalol-fluoroglucinol isomerasa (Krumholz & Bryant, 1988) y éste en dihidrofluoroglucinol por la acción de una fluoroglucinol reductasa (Brune *&* Schink, 1990; Brune *et al*., 1992). El dihidrofluoroglucinol se convierte en 3-hidroxi-5-oxohexanoato (HOHN) por la acción de una hidroxifluoroglucinol hidrolasa. Posteriormente el HOHN puede seguir

diferentes vías degradativas. Se ha descrito que la bacteria *Pelobacter massiliensis* degrada el HOHN a triacetato por la acción de la HOHN deshidrogenasa dando lugar finalmente a acetil CoA mediante la acción secuencial de las enzimas triacetil-CoA transferasa, triacetato β-cetotiolasa, acetoacetil-CoA β-cetotiolasa, fosfotransacetilasa y acetato quinasa (Brune *et al*., 1992). Sin embargo en *Eubacterium oxidoreducens* se ha descrito que el HOHN-CoA resultante se transforma en acetato y butirato tras la participación secuencial de las enzimas β-hidroxibutiril-CoA deshidrogenasa, butiril-CoA deshidrogenasa, acetil-CoA acetil transferasa, enoil-CoA hidrasa, fosfato acetiltransferasa y acetato quinasa (Krumholz *et al*., 1987). En el metabolismo anaerobio, el resorcinol, otro producto de la degradación de los galotaninos, se transforma en acetil-CoA tras sufrir un proceso de reducción y una hidrólisis. Sin embargo, las bacterias del rumen no son capaces de metabolizar el resorcinol y lo excretan al medio (Murdiati *et al*., 2005).

De todas las enzimas implicadas en la biotransformación de taninos, la enzima tanasa tiene una importancia fundamental puesto que cataliza el primer paso en su biotransformación. Esta enzima cataliza la reacción de hidrólisis de los enlaces éster presentes en galotaninos, taninos complejos y ésteres del ácido gálico (Figura 6).

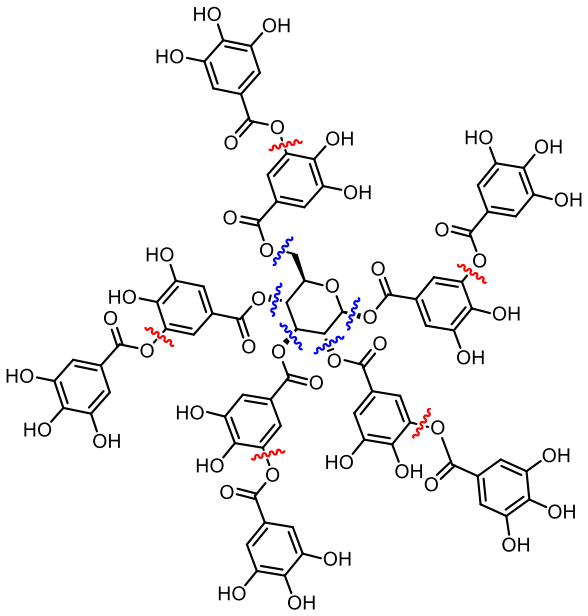

Figura 6. Actividad de la enzima tanasa sobre ácido tánico.

La enzima galato descarboxilasa es la encargada de catalizar la segunda reacción de biodegradación de galotaninos en la ruta anaerobia de degradación (Brune & Schink, 1990). Esta enzima cataliza la descarboxilación no oxidativa del ácido gálico (3,4,5-trihidroxibenzoato) produciendo pirogalol (1,2,3-bencenotriol) y liberando una molécula de dióxido de carbono (Figura 7). Este tipo de descarboxilación no oxidativa se caracteriza por la eliminación completa del grupo carboxilo del substrato, al contrario de lo que ocurre con la descarboxilación oxidativa, en la cual se sustituye el grupo carboxilo por un grupo hidroxilo (Chow *et al*., 1999).

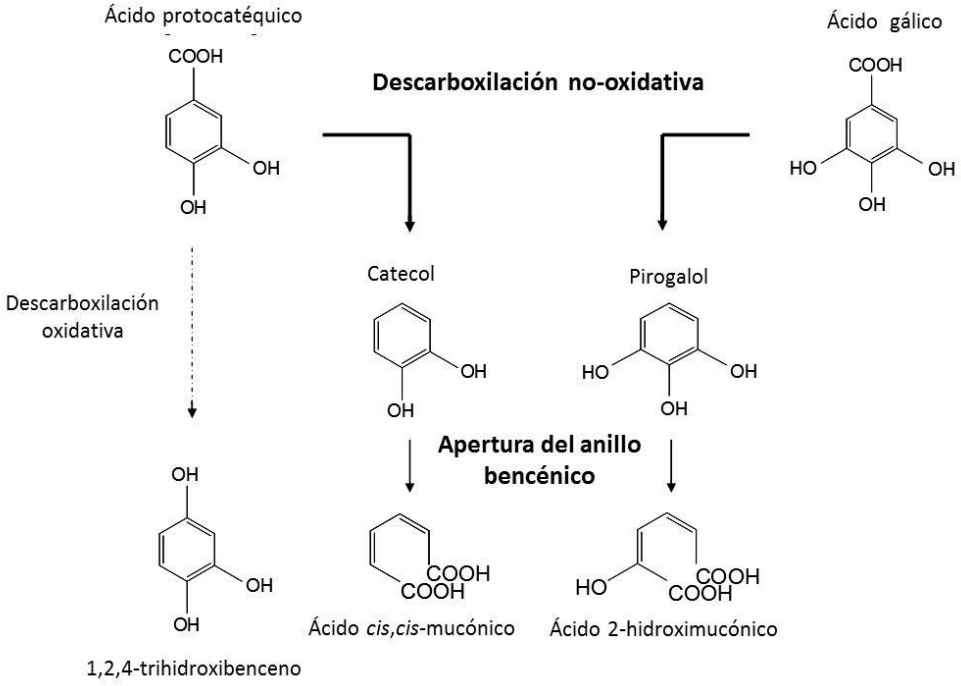

Figura 7. Ruta propuesta para la transformación de ácidos benzoicos en *A. adeninivorans*.

Se han descrito varios microorganismos que descarboxilan el ácido gálico de forma no oxidativa, como *P. agglomerans* (Zeida *et al*., 1998), *Enterococcus faecalis* (Nakajima *et al*., 1992), *Klebsiella pneumoniae* (Nakajima *et al*., 1992), *S. gallolyticus* (Chamkha *et al*., 2002) y *L. plantarum* (Osawa *et al*., 2000; Rodríguez *et al*., 2008b; Rodríguez *et al*., 2008c). Estas bacterias descarboxilan el ácido gálico hasta pirogalol finalizando así dicho metabolismo. La principal característica que presenta la enzima galato descarboxilasa es su sensibilidad al oxigeno (Haddock & Ferry, 1993; Nakajima *et al*., 1992; Imura & Hosono, 1996). Nakajima *et al*. (1992) purificaron la enzima galato descarboxilasa de *E. faecalis* y *K. pneumoniae* pero en estado inactivo, incluso en presencia de agentes estabilizantes. Zeida *et al*. (1998) purificaron

la enzima galato descarboxilasa de *P. agglomerans* y comprobaron que la proteína, una vez purificada perdía su actividad. A pesar de que se han descrito varias descarboxilasas del ácido gálico, la mayoría no han podido caracterizarse debido a su inestabilidad.

En la actualidad se han identificado y secuenciado algunas descarboxilasas de ácidos benzoicos, como la enzima 4-hidroxibenzoato descarboxilasa de *Salmonella typhimurium*, la enzima 4-hidroxibenzoato descarboxilasa de *Bacillus subtilis* o la enzima vainillato descarboxilasa de *Streptomyces* sp. D7. Todas ellas presentan una organización genética común caracterizada por la presencia de tres genes agrupados en una misma región (Lupa *et al.,* 2005) (Figura 8).

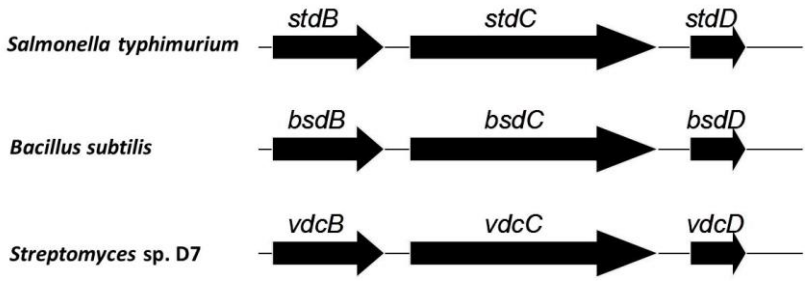

Figura 8. Genes implicados en la descarboxilación del ácido 4-hidroxibenzoico en *S. typhimurium* y *B. subtilis*, y en la descarboxilación del ácido vanillínico en *Streptomyces* sp. D7.

Para que las enzimas descarboxilasas del ácido 4-hidroxibenzoico de *S. typhimurium* y de *B. subtilis* y la enzima descarboxilasa del ácido vanillínico de *Streptomyces* sp. D7 sean activas es indispensable la presencia de los tres genes (Lupa *et al.,* 2005). Sin embargo Lupa *et al.* (2005) han observado en otros microorganismos organizaciones genéticas relacionadas con enzimas descarboxilasas de ácidos benzoicos constituidas por una agrupación de sólo dos genes. Estos resultados indican que posiblemente existen distintas organizaciones genéticas para las enzimas descarboxilasas de ácidos benzoicos. A pesar de estos estudios, todavía no se ha determinado la función de las proteínas codificadas por estos genes, de hecho, se ha descrito que en *Streptomyces* sp. D7, el gen *vdcD* codifica una proteína con un tamaño demasiado pequeño (9 kDa) como para ser una unidad catalítica (Chow *et al.,* 1999).

3.2. *Lactobacillus plantarum*: modelo de bacteria láctica degradadora de taninos

Las bacterias lácticas o bacterias del ácido láctico (BAL) representan un grupo heterogéneo de bacterias que producen ácido láctico como metabolito mayoritario de la fermentación de azúcares (Stiles & Holzapfel, 1997). Las BAL se asocian con hábitats ricos en nutrientes como suelos, forraje, estiércol, etc y algunas BAL se localizan preferentemente en la cavidad oral, tracto intestinal o vagina (Holzapfel *et al*., 2001). La mayoría de las BAL se consideran inocuas y poseen el estatus GRAS (*Generally Recognized As Safe*) para el consumidor (Tripathi *et al*., 2012).

Las BAL se encuentran presentes en una gran variedad de substratos vegetales y productos agroalimentarios en los que los compuestos fenólicos son abundantes. *L. plantarum* es el modelo de especie de BAL utilizada como cultivo iniciador en la fermentación de productos alimentarios de origen vegetal. Sin embargo, a pesar de la importancia de los compuestos fenólicos en la salud del consumidor y de la importancia de *L. plantarum* y su larga tradición de uso en la industria alimentaria, son escasos los estudios realizados sobre el metabolismo de los compuestos fenólicos en *L. plantarum* y en BAL en general.

En la tabla 1 y figura 9 se muestra un resumen del metabolismo de compuestos fenólicos descrito en *L. plantarum*.

Tabla 1. Metabolismo de compuestos fenólicos por *L. plantarum*.

Compuesto probado	Compuesto producido	Enzimas implicadas		Referencias
Ácido tánico	Ácido gálico	Tanasa	Lp_2956	Osawa *et al.,* 2000 Vaquero *et al.,* 2004 Curiel *et al*., 2009
Ácido gálico	Pirogalol	Descarboxilasa	Lp_2945	Rodríguez *et al.,* 2009
Pirogalol	No degradado			Rodríguez *et al.,* 2009
Ácido cafeico	*p*-Vinil catecol	PAD	Lp_3665	Cavin *et al.,* 1997a
	p-Etil catecol	Reductasa		Cavin *et al.,* 1997b Barthelmebs *et al.,* 2000 Rodríguez *et al.,* 2009
Catecol	No degradado			Whiting y Coggins, 1971 Rodríguez *et al.,* 2009
Ácido cinámico	No degradado			Rodríguez *et al.,* 2009
Ácido ferúlico	*p*-Vinil guaiacol	PAD	Lp_3665	Cavin *et al.,* 1997a
	p-Etil guaiacol	Reductasa		Cavin *et al.,* 1997b Rodríguez *et al.,* 2009
Ácido *m*-cumárico	Ácido 3-(3 hidroxifenil) propiónico	Reductasa		Rodríguez *et al.,* 2009
Ácido *o*-cumárico	No degradado			Rodríguez *et al.,* 2009
Ácido *p*-cumárico	*p*-Vinil fenol	PAD	Lp_3665	Cavin *et al.,* 1997a
	p-Etil fenol	Reductasa		Cavin *et al.,* 1997b Barthelmebs *et al.,* 2000 Rodríguez *et al.,* 2009
Ácido benzoico	No degradado			Rodríguez *et al.,* 2009
Ácido gentísico	No degradado			Rodríguez *et al.,* 2009
Ácido *p*-hidroxibenzoico	No degradado			Rodríguez *et al.,* 2009
Ácido protocatéquico	Catecol	Descarboxilasa	Lp_2945	Rodríguez *et al.,* 2009
Ácido quínico	Catecol	Varias enzimas		Whiting y Coggins, 1971 Whiting y Coggins, 1974 Whiting, 1975
Ácido salicílico	No degradado			Rodríguez *et al.,* 2009
Ácido sinápico	No degradado			Rodríguez *et al.,* 2009
Ácido siquímico	Catecol	Varias enzimas		Whiting y Coggins, 1971 Whiting y Coggins, 1974 Whiting, 1975
Ácido siríngico	No degradado			Rodríguez *et al.,* 2009
Ácido vanillínico	No degradado			Rodríguez *et al.,* 2009
Ácido verátrico	No degradado			Rodríguez *et al.,* 2009
Oleuropeina	Aglicona	β-glucosidasa		Marsilio *et al.,* 1996
Aglicona de oleuropeina	Hidroxitirosol Ácido elenoico	Esterasa		Marsilio *et al.,* 1996
Ferulato de metilo	Ácido ferúlico	Feruloil esterasa	Lp_0796	Esteban-Torres *et al*., 2013
Acetato de fenilo	Fenol	Aril esterasa	Lp_1002	Esteban Torres *et al*., 2014

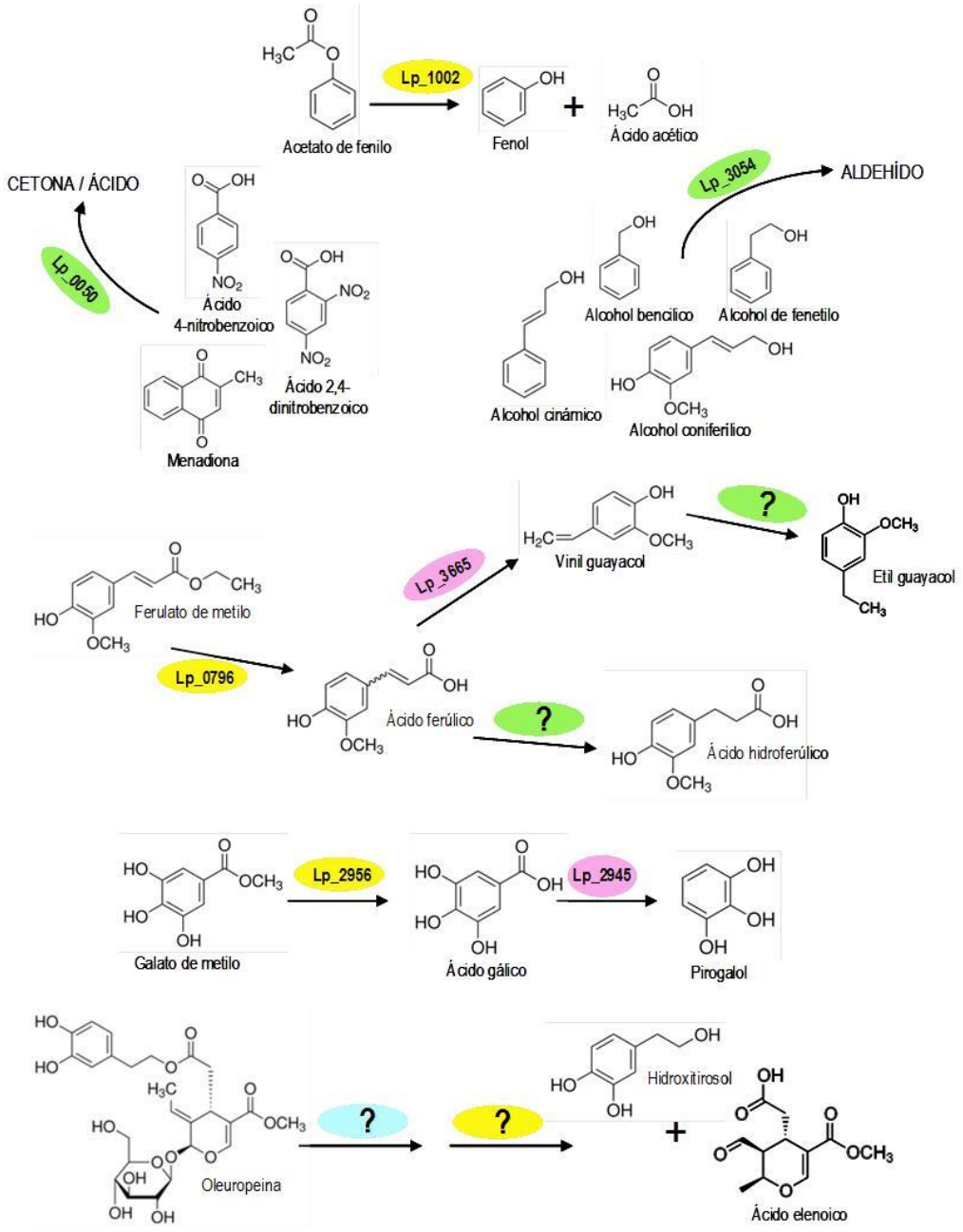

Figura 9. Metabolismo de compuestos fenólicos en *L. plantarum*. Las enzimas con actividad reductasa se encuentran marcadas en color verde, esterasa en amarillo, β-glucosidada en azul y descaroxilasa en rosa.

En los últimos años se ha estudiado la capacidad que presenta *L. plantarum* para degradar galotaninos. Osawa *et al*. (2000) aislaron, a partir de alimentos fermentados, cepas de *L. plantarum* que poseían actividades tanasa y galato descarboxilasa. Posteriormente, Vaquero *et al*. (2004) confirmaron la presencia de actividad tanasa en este microorganismo en cepas aisladas de vinos. Rodríguez *et al*. (2007) describieron que, de manera general, *L. plantarum* degrada galotaninos mediante la despolimerización de taninos de alto peso

molecular y una reducción de los taninos de bajo peso molecular, originando ácido gálico y pirogalol como metabolitos finales. *L. plantarum* degrada galotaninos, como el ácido tánico, mediante una ruta de degradación anaerobia que implica la presencia de dos actividades enzimáticas sucesivas. Inicialmente, la enzima tanasa, o esterasa de ácido gálico, actúa sobre el ácido tánico produciendo ácido gálico, y posteriormente, mediante una descarboxilación no-oxidativa catalizada por la enzima galato descarboxilasa, se origina pirogalol a partir del ácido gálico formado (Figura 10) (Rodríguez *et al*., 2008b).

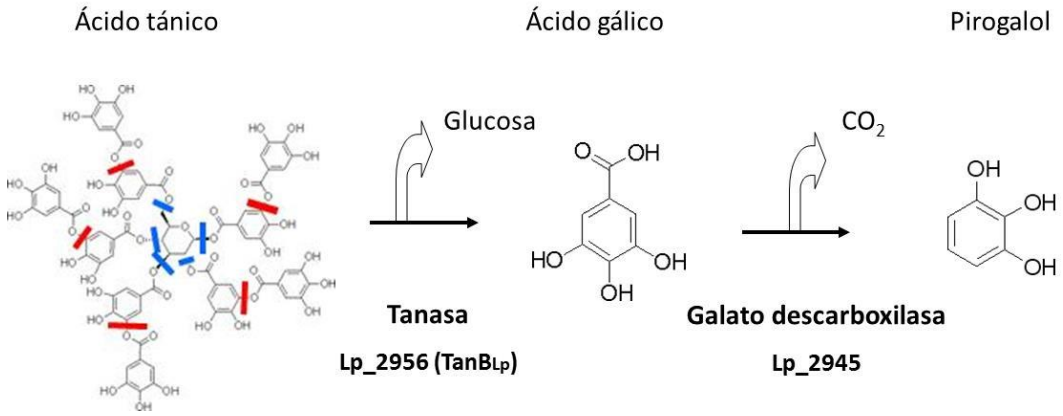

Figura 10. Mecanismo de degradación del ácido tánico por *L. plantarum.*

La enzima tanasa de *L. plantarum* (TanB$_{Lp}$, TanLp1 o Lp_2956) se identificó por Iwamoto *et al*. (2008) debido a que presentaba un 27% de identidad con la enzima tanasa TanA$_{Sl}$ de *Staphylococcus lugdunenis* previamente descrita (Noguchi *et al*., 2007). Curiel (2010) describió que la proteína Lp_2945, anotada como 3-octaprenil-4-hidroxibenzoato descarboxilasa o UbiD en el genoma de *L. plantarum* WCFS1, estaba implicada en la actividad galato descarboxilasa, puesto que se inducía en presencia de ácido gálico y su interrupción originaba la desaparición de la actividad enzimática (Curiel, 2010).

La repuesta de *L. plantarum* a la presencia de galotaninos se ha estudiado desde distintas aproximaciones experimentales. Mediante estudios proteómicos se ha evaluado el efecto del ácido tánico en dos cepas de *L. plantarum*, WCFS1 y VP08. Los resultados indican que la cepa VP08, aislada de vino, responde alterando los niveles de proteínas involucradas en la glicolisis, metabolismo de aminoácidos, traducción y plegamiento de proteínas (Cecconi *et al*., 2009) mientras que en la cepa WCFS1, aislada del TGI superior, se observa la modificación

en los niveles de la síntesis de proteínas relacionadas con el biogénesis de pared, defensa contra estrés oxidativo y ahorro de energía, así como también un incremento en la proteína Lp_2945, implicada en la actividad galato descarboxilasa (Curiel *et al*., 2011; Curiel, 2010). Reverón *et al*. (2013) estudiaron la influencia del ácido tánico en la adaptación molecular de *L. plantarum* y observaron un aumento de la expresión de genes asociados a supervivencia en el tracto gastrointestinal, como *copA* y *lp_2940.* Además describieron que el gen que codifica la enzima tanasa $TanB_{Lp}$ (Iwamoto *et al*., 2008), incrementó sus niveles de expresión de forma dependiente a la concentración de ácido tánico (Reverón *et al*., 2013).

4. ENZIMA TANASA: APLICACIONES

La enzima tanasa, responsable de la degradación de galotaninos, es una enzima con numerosas aplicaciones industriales. Las principales aplicaciones de la enzima tanasa están relacionadas con la producción de té instantáneo, zumos de frutas, así como producción de ácido gálico (Chávez-González *et al*., 2012; Aguilar *et al*., 2007). El tratamiento enzimático con tanasa para la producción de té instantáneo mejora su solubilidad en agua fría y evita la formación de precipitados (Kumar *et al*., 2011). Estos precipitados se forman por una polimerización de polifenoles esterificados, la enzima tanasa hidroliza estos enlaces éster y evita así la precipitación de los mismos (Sanderson *et al.,* 1974).

También se utiliza la enzima tanasa durante el procesado de bebidas y alimentos ricos en taninos para mejorar la calidad final del producto (Boadi *&* Neufeld, 2001). Los zumos de frutas contienen un alto contenido en taninos responsables del amargor del zumo y de su astringencia, además afectan al color y provocan turbidez y sedimentos. El tratamiento con la enzima tanasa contribuye a reducir el amargor en zumos de frutas, disminuyendo su turbidez y aumentando su calidad.

Otra importante aplicación de la enzima tanasa es la producción de ácido gálico y galato de propilo, a partir de residuos industriales con alto contenido en taninos. El ácido gálico se utiliza en la industria farmacéutica como compuesto intermediario en la síntesis de trimetropina (Chávez- González *et al*., 2012). Además, a partir del ácido gálico obtenido se pueden obtener pirogalol y otros ésteres del ácido gálico, como el galato de propilo, los cuales se utilizan en la industria alimentaria como conservantes y antioxidantes (Yu *et al*., 2004). La enzima tanasa también se usa para el tratamiento de piensos animales, puesto que interviene

en los procesos de eliminación de taninos en los cereales utilizados en la dieta animal (Nuero *&* Reyes, 2002).

Las enzimas tanasas de hongos son las más estudiadas. Los hongos producen la enzima tanasa mayoritariamente de forma extracelular, sin embargo también se ha observado una pequeña producción intracelular (Bradoo *et al.,* 1997; Bajpai *&* Patil, 1996; Bhat *et al.,* 1998). De manera general, las tanasas fúngicas presentan una estabilidad a pHs entre 3.5-8.0, siendo su pH óptimo 5.0-6.0. Las proteínas son estables entre 20-60 ºC, teniendo su temperatura óptima alrededor de 30-40 ºC (Belmares *et al.,* 2004). Los cationes Mg^{2+} y Hg^{+} incrementan la actividad de la enzima tanasa, mientras que los cationes Ba^{2+}, Zn^{2+}, Hg^{2+}, Ag^{+}, Fe^{3+} y Co^{2+} inhiben por completo su actividad (Kar *et al.,* 2003). Además el EDTA y el β-mercaptoetanol también inactivan la enzima. Los estudios de especificidad de substrato demuestran que poseen actividad frente a substratos como ácido tánico o galato de metilo. Pero también se ha descrito que algunas tanasas fúngicas degradan otros substratos como por ejemplo el galato de epigalocatequina (*Verticillium* sp*., Apergillus ficuum, A. oryzae y A. niger*), galato de galocatequina (*A. niger*) o el galato de propilo (*A. niger* y *A. oryzae*).

Respecto a las enzimas tanasas de origen bacteriano, la enzima $TanA_{Sl}$ de *Staphylococcus lugdunensis* sólo se ha identificado genéticamente (Mondal *et al.,* 2001) mientras que la enzima tanasa $TanB_{Lp}$ de *L. plantarum* ATCC 14917^{T} también se ha caracterizado bioquímicamente (Iwamoto *et al*., 2008; Curiel *et al*., 2009). La enzima tanasa $TanB_{Lp}$ de *L. plantarum* ATCC 14917^{T} además del galotanino ácido tánico, degrada los ésteres derivados de los ácidos gálico y protocatéquico, así como los galatos de catequina, epicatequina, galocatequina y galoepicatequina (Curiel *et al*., 2009). La enzima $TanB_{Lp}$ posee un pH y temperatura óptimos de pH 8.0 y 40 ºC respectivamente (Iwamoto *et al.,* 2008). Previamente Rodríguez *et al.* (2008a) caracterizaron la actividad tanasa presente en extractos proteicos de *L. plantarum* CECT 748^{T} (ATCC 14917^{T}) y describieron que la actividad tanasa presentó unos valores óptimos de pH 5.0 y 30 ºC de temperatura. Los resultados obtenidos con extractos celulares de *L. plantarum* CECT 748^{T} (ATCC 14917^{T}) difieren de los obtenidos para la enzima tanasa $TanB_{Lp}$ purificada a partir de la misma cepa, lo que puede sugerir la existencia de una segunda enzima tanasa en los extractos proteicos de *L. plantarum* CECT 748^{T} (ATCC 14917^{T}) (Iwamoto *et al.,* 2008).

Objetivos

Los taninos son constituyentes importantes de los alimentos de origen vegetal y están directamente relacionados con las características sensoriales de los mismos por lo que tienen una importancia indiscutible en tecnología de alimentos. Además, la presencia de taninos en la dieta afecta a la salud del consumidor de manera antagónica, con demostrados efectos beneficiosos (antioxidante, anticarcinogénico, etc) y efectos adversos (prooxidante, mutagénico, etc.). En el TGI humano existe un ecosistema complejo de microorganismos en el que se encuentran bacterias capaces de metabolizar taninos, modulando tanto su absorción como su actividad, lo que influye finalmente en la salud del consumidor. Respecto al metabolismo de taninos, en bacterias se ha descrito la presencia de enzimas tanasas capaces de hidrolizar galotaninos dando lugar a ácido gálico. *S. lugdunensis*, patógeno humano, y *L. plantarum*, presente en el TGI humano y en la fermentación de alimentos de origen vegetal, poseen los genes $tanA_{Sl}$ y $tanB_{Lp}$, respectivamente, que codifican enzimas tanasas. Además, las cepas de *L. plantarum* son capaces de transformar el ácido gálico en pirogalol por acción de una enzima con actividad galato descarboxilasa. La realización de búsquedas en las bases de datos de proteínas similares a las tanasas descritas permitirá conocer bacterias presentes en el TGI humano que poseen esta capacidad metabólica.

Teniendo en cuenta estos antecedentes y con objeto de conocer la presencia en el TGI humano de bacterias capaces de degradar galotaninos se han propuesto los siguientes objetivos:

1. Estudio del metabolismo de galotaninos en bacterias cultivables presentes en el TGI humano.

2. Conocer las características bioquímicas de las proteínas tanasas presentes en estos microorganismos.

Capítulos

Capítulo 1

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Contribution of a tannase from *Atopobium parvulum* DSM 20469^T in the oral processing of food tannins

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ABSTRACT

During oral passage, food tannins interact with the microbiota present in the oral cavity. *Atopobium parvulum* strains are inhabitants of the human oral cavity. A gene encoding a protein similar to bacterial tannases is present in *A. parvulum* strains. The *tanA*$_{Ap}$ (*apar_1020*) gene was cloned and expressed in *Escherichia coli* BL21 (DE3). The overproduced TanA$_{Ap}$ protein was purified to homogeneity. It exhibited optimal activity at pH 6.0 and broad temperature range, being these properties compatible with its action during food oral processing. However, purified TanA$_{Ap}$ protein presented the lowest specific activity among bacterial tannases (3.5 U/mg) and was unable to hydrolyze complex tannin, such as tannic acid. These biochemical properties discard a main role of TanA$_{Ap}$ in the breakdown of complex food tannins during oral processing.

Introduction

Vegetable tannins are abundant in plants utilized as human food. Tannins occur widely on common foodstuffs, such as pomegranate, banana, strawberry, grape, cashew nut, and hazelnut. Drinks like wine and tea also contain these phenolic compounds (Shahidi & Naczk, 2003). The molar mass of tannins affects tannin characteristics directly, and it has been suggested that small molecule tannins have more antioxidant activity (Ordoudi & Tsimidou, 2006). To understand the biological effects of food tannins the insight on the metabolic fate and bioavailability of these metabolites in the human body is crucial, however this knowledge is currently scarce (Moco, Martin, & Rezzi, 2012; Rechner, Kuhnle, Bremner, & Hubbard, 2002). The interaction of tannins with human microbiota will determine in great extent the physiological effects of these polyphenols.

Although much work has been focused on factors that determine mechanical (e.g. rheological and fracture) and sensory properties of foods, far less attention has been paid to linking food transformations that occur during oral processing with microbial action (Chen, 2009). The human microbiome is a dynamic community changing in response to natural perturbations such as diet (Spencer et al., 2011; Turnbaugh et al., 2009; Wu et al., 2011; Zhang et al., 2010). The oral cavity of humans hosts several hundred taxa, with remarkable diversity even among saliva, tongue, teeth, and other substrates (Dewhirst et al., 2010; Segata et al., 2012). All of the surfaces of the mouth are covered in a bacterial biofilm (Wade, 2013). Studies on the tongue biofilm have been relatively few in number, compared with the significant number of investigations of dental plaque and the microbiota associated with periodontal disease and dental caries. The tongue is known to harbor a very diverse microbiota at high cell density. Among the many bacteria present in the oral cavity, the species *Atopobium parvulum* is of interest because its members are frequently isolated from the human oral cavity, especially from the tongue (Copeland et al., 2009; Riggio et al., 2008). The genome of *A. parvulum* type strain (IPP 1246^T) has been completely sequenced. An ORF (*apar_1020*) encoding a "putative uncharacterized protein" had 40% and 26% identity to TanA$_{Sl}$, a tannase from *Staphylococcus lugdunensis*, and TanB$_{Lp}$ (formerly called TanLp1), a tannase from *Lactobacillus plantarum*, respectively.

The enzyme tannase (or tannin acyl hydrolase EC 3.1.1.20) belongs to the serine esterases, catalyzing the hydrolysis of the ester bond (galloyl ester of an alcohol moiety) and depside bond (galloyl ester of gallic acid) in tannins to release gallic acid (Aguilar et al., 2007; Chávez-González et al., 2012). Tannase activity contributes to the hydrolysis of natural tannins present in the diet. Although bacteria possessing tannase activity, such as *L. plantarum*, *Streptococcus gallolyticus*, and *S. lugdunensis*, have been described in the human gastrointestinal tract (Abdulamir, Hafidh, & Bakar, 2011; Abdulamir, Hafidh, Mahdi, Al-Jeboori, & Abubaker, 2009; Noguchi et al., 2007; Rusniok et al., 2010), there are still many questions about the oral metabolism of food tannins. It will be interesting to know if during the short period of oral processing, tannin hydrolysis began in the mouth. The presence of a protein similar to bacterial tannases in *A. parvulum*, a species abundant in the oral cavity, will be important for predicting their contribution to food tannin breakdown. Therefore, the objective of this study was to find out the potential contribution of TanA$_{Ap}$ (Apar_1020) protein from *A. parvulum* to tannin hydrolysis during food oral processing.

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Materials and methods

Strains, plasmids, and materials

A. parvulum DSM 20469^T (IPP 1246^T, ATCC 33793^T) used through this study was purchased from the DSM (German Collection of Microorganisms and Cell Cultures). *Escherichia coli* DH10B was used as host strain for all DNA manipulations. *E. coli* BL21 (DE3), providing a T7 RNA polymerase, was used for heterologous expression in the pURI3-Cter vector (Curiel, de las Rivas, Mancheño, & Muñoz, 2011). *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 °C and shaking at 200 rpm. When required, ampicillin was added to the medium at a concentration of 100 μg/ml.

Plasmid DNA was extracted by a High Pure plasmid isolation kit (Roche). PCR product was purified with a QIAquick gel extraction kit (Quiagen). Oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany). *Dpn*I and Prime STAR HS DNA polymerase were obtained from Takara. His-tagged protein was purified by a Talon Superflow resin (Clontech). The compounds assayed in the study were methyl gallate (Fluka), ethyl gallate (Aldrich), propyl gallate (Sigma), lauryl gallate (Aldrich), ethyl protocatechuate (ethyl 3,4-dihydroxybenzoate) (Aldrich), and tannic acid (Sigma).

Cloning of $TanA_{Ap}$

The gene encoding for a putative tannase (*apar_1020*, or $tanA_{Ap}$) in *A. parvulum* DSM 20469^T (accession YP_003180040) was amplified by PCR by using the primers 1394 (5′-*ACTTTAAGAAGGAGATAT ACATat*gtctgataatacgaatcaacctgca) and 787 (5′-*GCTATTAATGATGATG ATGATGATG*agcagacgcacacgagacaatcca) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the $tanA_{Ap}$ gene sequence are written in lowercase letters). As a peptide signal was predicted in the $TanA_{Ap}$ sequence, oligonucleotides 1394 and 787 were used to clone $TanA_{Ap}$ lacking the 23-amino acid peptide signal sequence. Prime Star HS DNA polymerase (Takara) was used for the PCR amplification. The 1.7-kb purified PCR product was inserted into the pURI3-Cter vector using a restriction enzyme- and ligation-free cloning strategy (Curiel et al., 2011). The vector produces recombinant proteins having a six-histidine affinity tag in their C-termini. *E. coli* DH10B cells were transformed, recombinant plasmids were isolated, and those containing the correct insert were identified by size, verified by DNA sequencing, and then transformed into *E. coli* BL21 (DE3) cells for expression.

Enzyme production and purification

E. coli BL21 (DE3) harboring the recombinant plasmid pURI3-Cter-$TanA_{Ap}$ was grown in LB medium containing 100 μg/ml ampicillin on a rotary shaker (200 rpm) at 37 °C until an optical density (OD) at 600 nm of 0.4 was reached. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM and protein induction was continued at 22 °C during 18 h.

The induced cells were harvested by centrifugation (8000 *g*, 15 min, 4 °C), resuspended in phosphate buffer (50 mM, pH 6.5) and disrupted by French Press passages (three times at 1100 psi). The insoluble fraction of the lysate was removed by centrifugation at 47,000 *g* for 30 min at 4 °C, and the supernatant was filtered through a 0.2 μm pore-size filter and then loaded onto a Talon Superflow resin (Clontech) equilibrated in phosphate buffer (50 mM, pH 6.5) containing 300 mM NaCl and 10 mM imidazole to improve the interaction specificity in the affinity chromatography step. The bound enzyme was eluted using 150 mM imidazole in the same buffer. The purity of the enzyme was determined by SDS-PAGE in Tris-glycine buffer. Fractions containing the His6-tagged protein were pooled and analyzed for tannase activity.

Enzyme activity assay

Tannase activity was determined using a colorimetric assay using rhodanine, specific for gallic acid (Inoue & Hagerman, 1988). Rhodanine reacts only with gallic acid and not with galloyl esters or other phenolics. Gallic acid analysis in the reactions was determined using the following colorimetric assay. Tannase enzyme (100 μg) in 700 μl of 50 mM phosphate buffer pH 6.5 was incubated with 40 μl of 25 mM methyl gallate (1 mM final concentration) during 5 min at 37 °C. After this incubation, 150 μl of a methanolic rhodanine solution (0.667% w/v rhodanine in 100% methanol) was added to the mixture. After 5 min incubation at 30 °C, 100 μl of 500 mM KOH was added. After an additional incubation of 5–10 min, the absorbance at 520 nm was measured on a spectrophotometer. A standard curve using gallic acid concentration ranging from 0.125 to 1 mM was prepared. One unit of tannase activity was defined as the amount of enzyme required to release 1 μmol of gallic acid per minute under standard reaction condition.

Determination of pH and temperature effects on tannase activity

The optimum pH value of $TanA_{Ap}$ was determined by measuring its activity at different pH values (3.0–10.0). The following buffers all at 100 mM were used for the assay: acetic acid–sodium acetate (pH 3.0–5.0), citric acid–sodium citrate (pH 6), sodium phosphate (pH 7), Tris–HCl (pH 8), glycine–NaOH (pH 9), and sodium carbonate–bicarbonate (pH 10). The rhodanine assay was used for the optimal pH characterization of tannase. Since the rhodanine–gallic acid complex forms only in basic conditions, after the completion of the enzymatic degradation of methyl gallate, KOH was added to the reaction mixture to ensure that the same pH value (pH 11) was achieved in all samples assayed. Determinations were done in triplicate.

The optimum temperature of $TanA_{Ap}$ was assayed by incubating the purified protein in 25 mM phosphate buffer (pH 6.5) at seven different temperatures in the range of 4–65 °C (4, 22, 30, 37, 45, 55 and 65 °C). To study the thermal stability of $TanA_{Ap}$, tannase was incubated at temperatures over the range of 22–65 °C (22, 30, 37, 45, 55 and 65 °C) for 30 min and 2, 4, 6, and 18 h. Aliquots were withdrawn in triplicate at these incubation times to test the remaining activity at standard conditions. The non-heated enzyme was considered as control (100%).

Effect of metal ions, reagents, and inhibitors on tannase activity

The effect of various metal ions (Mg^{2+}, K^+, Ca^{2+}, Hg^{2+}, and Zn^{2+}), metal chelator EDTA, surfactants (SDS, Tween 80, and Triton X-100), and other reagents (DMSO, and β-mercaptoethanol) on tannase activity was investigated by the rhodanine assay using methyl gallate as substrate. Purified $TanA_{Ap}$ was incubated with additives (1 mM final concentration) at 30 °C for 1 h. After incubation, the residual activity was measured in triplicate under the standard assay conditions. The relative activities were calculated with respect to the control where the reaction was carried out in the absence of additives (100%).

HPLC-DAD analysis of substrate specificity

The substrate specificity of $TanA_{Ap}$ was determined using five commercial hydroxybenzoic esters (methyl gallate, ethyl gallate, propyl gallate, lauryl gallate, and ethyl protocatechuate), and a hydrolyzable tannin (tannic acid). The standard enzyme assay was modified by using 200 μg of $TanA_{Ap}$, and 1 mM substrate, in the reaction mixture and incubated at 37 °C during 10 min. As controls, phosphate buffer containing the reagents but the enzyme were incubated in the same conditions.

The reaction products were extracted twice with ethyl acetate (Lab-Scan, Ireland) and analyzed by HPLC-DAD. A Thermo (Thermo Electron Corporation, Waltham, Massachusetts, USA) chromatograph equipped with a P400 SpectraSystem pump, AS3000

autosampler, and a UV6000LP photodiode array detector was used. A gradient of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 78:20:2, v/v/v) was applied to a reverse-phase Nova-pack C_{18} (25 cm × 4.0 mm i.d.) 4.6 μm particle size, cartridge at room temperature as follows: 0–55 min, 80% B linear, 1.1 ml/min; 55–57 min, 90% B linear, 1.2 ml/min; 57–70 min, 90% B isocratic, 1.2 ml/min; 70–80 min, 95% B linear, 1.2 ml/min; 80–90 min, 100% linear, 1.2 ml/min; 100–120 min, washing 1.0 ml/min, and reequilibration of the column under initial gradient conditions. Detection was performed by scanning from 220 to 380 nm. Samples were injected onto the cartridge after being filtered through a 0.45 μm PVDF filter. The identification of degradation compounds was carried out by comparing the retention times and spectral data of each peak with those of standards from commercial suppliers.

Results and discussion

Production and characterization of purified TanA$_{Ap}$

Tannases are capable of hydrolyzing complex tannins, which represent an important chemical group occurring in food plants. Studies related to tannase-producing strain isolation have been conducted (Aguilar et al., 2007; Chávez-González et al., 2012). Among the many bacteria present in the oral cavity, *A. parvulum* strains are of interest because they possess a gene putatively encoding a tannase. The *apar_1020* (*tanA$_{Ap}$*) gene predicted to encode a 607 amino acid protein 39.5% identical to TanA$_{Sl}$, a tannase from *S. lugdunensis*, and 26% identical to TanB$_{Lp}$, a tannase from *L. plantarum*, the only two bacterial tannases genetically described so far (data not shown). Noteworthy, TanA$_{Sl}$ and TanB$_{Lp}$ are only 27% identical among them (Iwamoto, Tsuruta, Nishitaini, & Osawa, 2008). In TanA$_{Ap}$ a signal peptide was predicted with a cleavage site at residue 23. Therefore, processed mature TanA$_{Ap}$ protein has 584 amino acid residues, with a predicted molecular mass of 63.8 kDa, and an isoelectric point of 4.62. As TanA$_{Ap}$ showed high amino acid identity to bacterial tannases, the tannase activity of TanA$_{Ap}$ needs to be assayed.

The *tanA$_{Ap}$* gene was cloned into the pURI3-Cter expression vector by a ligation-free cloning strategy described previously (Curiel et al., 2011). The vector incorporates the DNA sequence encoding hexahistidine to create a His-tagged fusion enzyme for further purification step. The integrity of the construct was confirmed by DNA sequencing. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) and expressed under the control of an inducible IPTG promoter. Cell extracts were used to detect the presence of overproduced proteins. SDS-PAGE analysis showed that there was one major band of protein, approximately 66 kDa, in the intracellular soluble fraction of the pURI3-Cter-TanA$_{Ap}$ cells, which was absent in the control pURI3-Cter cells (Fig. 1). The molecular weight of the overproduced protein was consistent with the calculated molecular weight of TanA$_{Ap}$. Since the cloning strategy would yield a His-tagged protein variant, *A. parvulum* pURI3-Cter-TanA$_{Ap}$ could be purified on an immobilized metal affinity chromatography (IMAC) resin. The recombinant protein was observed as single band on SDS-PAGE (Fig. 1). Routinely about 12 mg of purified protein from 1-liter culture was obtained.

TanA$_{Ap}$ protein purified by the affinity resin was biochemically characterized. A method specific for the detection of gallic acid could be used for a reliable quantification of tannase activity. Since tannase catalyzes the hydrolysis of the galloyl ester linkage liberating gallic acid, the activity of tannase could be measured by estimating the gallic acid formed due to enzyme action (Mueller-Harvey, 2001). Inoue and Hagerman (1988) described a rhodanine assay specific for determining free gallic acid. Rhodanine reacts with gallic acid to give a red complex with a maximum absorbance at 520 nm. Rhodanine assay was used to determine the specific activity of TanA$_{Ap}$, simultaneously, the activity of the previously described TanB$_{Lp}$ tannase from *L. plantarum* was also determined as reference. Using methyl gallate as substrate, the specific activity of TanA$_{Ap}$ purified enzyme was 3.5 U/mg, 116 times lower than that of TanB$_{Lp}$ (408 U/mg). This low specific activity could indicate that even though tannase action from *A. parvulum* could begin almost immediately after food ingestion, its contribution to tannin breakdown might not be relevant.

In relation to the biochemical properties of the enzyme, TanA$_{Ap}$ showed optimal activity at pH 6 (Fig. 2A), slightly more acidic than the optimal pH for TanB$_{Lp}$ (pH 7). During food oral processing, saliva provides buffering effects. It was indicated that the pH of saliva rises during the first 5 min after the intake of most foods, and falls to around 6, or lower, approximately 15 min after food consumption (Humphrey & Williamson, 2001). Therefore, TanA$_{Ap}$ could found an adequate pH for activity during food oral processing.

Despite the optimum temperature of TanA$_{Ap}$ was 55 °C, at 37 °C, the physiological temperature for humans, 80% of the maximal activity was found. Similarly, more that 80% maximal activity was obtained at 20, 42, and 65 °C (Fig. 2B). Tannase TanB$_{Lp}$ from *L. plantarum* showed maximal activity at 40 °C, having only 50% of the maximal activity at 30 or 60 °C. The thermal stability profile for TanA$_{Ap}$ is shown in Fig. 2C. According to the thermal stability profile, TanA$_{Ap}$ was most stable at temperatures between 37 and 65 °C, and more than 60% enzyme activity remained after 18 h at 45 °C (Fig. 2C). Tannase TanB$_{Lp}$ from *L. plantarum* kept less than 20% of the maximal activity after incubation at 37 °C during 20 h. The data for TanA$_{Ap}$ demonstrated that the enzyme exhibited high thermal stability under prolonged incubation up to 45 °C. Despite the specific activity of TanA$_{Ap}$ is remarkably lower than the activity of TanB$_{Lp}$, TanA$_{Ap}$ is more thermostable, and therefore it is able to resist thermal unfolding in the absence of its substrate.

The effects of several ions and additives are shown in Table 1. Contrarily to TanB$_{Lp}$, $CaCl_2$ did not activate TanA$_{Ap}$ (Curiel et al., 2009). Tannase activity of TanA$_{Ap}$ was not increased by any of the additives assayed. Similarly to TanB$_{Lp}$, activity was greatly inhibited by β-mercaptoethanol and by the metal ion Hg^{2+} (Curiel et al., 2009). $ZnCl_2$ significantly inhibited TanA$_{Ap}$ activity (relative activity 24%). The other metal ions and additives assayed partially affected tannase activity (relative activity 74–96%). The different additive effect observed would suggest that there are notable structural differences among both bacterial tannases, TanA$_{Ap}$ from *A. parvulum*, and TanB$_{Lp}$ from *L. plantarum*.

Despite the low specific activity showed by TanA$_{Ap}$, this protein possesses biochemical properties compatible with its action during food oral passage, since its pH and temperature for activity are provided by the human saliva during food processing. Oral processing occurs during a short time period, however, it has been described that during the short period of oral processing, about 50% of bread and 25% of pasta starch are hydrolyzed and transformed into smaller molecules by the amylase

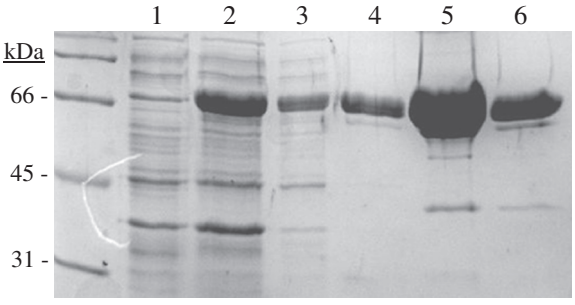

Fig. 1. Purification of TanA$_{Ap}$ tannase from *A. parvulum*. Analysis by SDS-PAGE of soluble cell extracts of IPTG-induced *E. coli* BL21 (DE3) (pURI3-Cter) (1) or *E. coli* BL21 (DE3) (pURI3-Cter-TanA$_{Ap}$) (2), flow through (3), or fractions eluted after His affinity resin (4–6). The gel was stained with Coomassie blue. Molecular mass markers are located at the left (SDS-PAGE Standards, Bio-Rad).

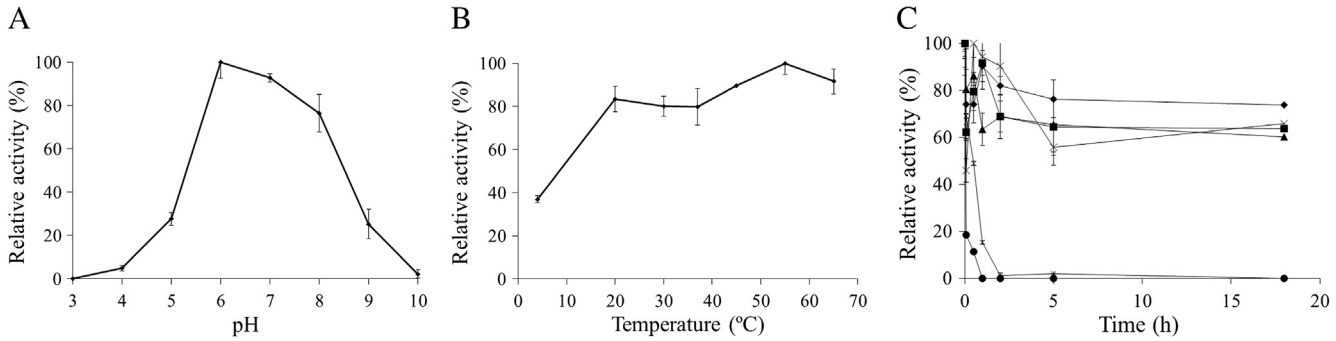

Fig. 2. Biochemical properties of $TanA_{Ap}$ protein. (A) pH-activity profile of $TanA_{Ap}$. (B) Temperature-activity profile of $TanA_{Ap}$. (C) Thermal stability profile for $TanA_{Ap}$ after preincubation at 22 °C (filled diamond), 30 °C (filled square), 37 °C (filled triangle), 45 °C (cross), 55 °C (star), and 65 °C (filled circle) in phosphate buffer (50 mM, pH 6.5); at indicated times, aliquots were withdrawn, and analyzed as described in the Materials and methods section. The experiments were done in triplicate. The mean value and the standard error are showed. The percentage of residual activity was calculated by comparing with unincubated enzyme.

enzyme present in the human saliva (Hoebler, Devaux, Karinthi, Belleville, & Barry, 2000; Hoebler et al., 1998). The interaction of amylase enzyme with starch ingredients produces almost an immediate effect on hydrolysis, and thus making the food intake much easily mixable and digestible in the stomach. A similar situation could be envisaged for the action of $TanA_{Ap}$ on the tannins present on the diet.

Contribution of $TanA_{Ap}$ to the hydrolysis of tannins from the diet

Tannins are natural polyphenolic compounds present in food plants. They are characterized by their ability to form strong complexes with different minerals and macromolecules, such as proteins, cellulose, and starch, causing astringency and precipitation effects (Mingshu, Kai, Quiang, & Dongying, 2006; Serrano, Puupponen-Pimiä, Dauer, Aura, & Saura-Calixto, 2009). As a result, tannins are considered antinutritional. Tannases catalyzes the hydrolysis reaction of ester bonds present in the gallotannins, complex tannins, and gallic acid and protocatechuic acid esters (Aguilar et al., 2007; Chávez-González et al., 2012; Curiel et al., 2009). In order to know the substrate specificity of $TanA_{Ap}$, several gallate and protocatechuate esters were assayed. As shown in Fig. 3, none of the esters assayed were significantly hydrolyzed. Methyl, ethyl, and propyl gallate and ethyl protocatechuate were minimally hydrolyzed. Lauryl gallate, possessing a long aliphatic alcohol chain, was not hydrolyzed at all (data not shown). Contrarily to these results, *L. plantarum* tannase ($TanB_{Lp}$) was able to fully hydrolyze gallic esters even those having an alcohol substituent as longer as lauryl (C12) (Curiel et al., 2009). Structural differences among both bacterial proteins will be responsible of the different spatial requirements observed for tannase activity.

Table 1
Effect of additives on *A. parvulum* tannase activity.

Additions (1 mM)	Relative activity (%)
Control	100
EDTA	74
KCl	80
$HgCl_2$	16
$CaCl_2$	97
$MgCl_2$	96
$ZnCl_2$	24
Triton X-100	83
DMSO	82
Tween 80	94
Urea	83
β-Mercaptoethanol	15

It is noteworthy to mention that the colorimetric rhodanine assay used for the detection and quantification of tannase activity is much more sensitive that the analysis of the reaction products by HPLC. By using methyl gallate as substrate, the rhodanine assay allowed to determine properly the biochemical properties of $TanA_{Ap}$ assayed; however, this characterization would not be possible by the HPLC analysis.

In order to evaluate the contribution of $TanA_{Ap}$ action during oral processing of diet tannins, a complex and natural tannin, tannic acid, was incubated in the presence of $TanA_{Ap}$. Tannic acid is almost exclusively formed by poly-galloyl glucose derivatives whose nature and complexity vary with the plant source. When $TanA_{Ap}$ was incubated on tannic acid, a hydrolysis profile identical to the control without enzyme was observed (data not shown). This was an expected result considering the minimal degradation on simple gallic acid esters observed after $TanA_{Ap}$ action. As $TanA_{Ap}$ did not show activity on tannic acid, it could be possible that the natural tannin substrate for this enzyme will be different and still remained unknown. In addition, specific reaction conditions or the presence of an unknown cofactor will be required to increase $TanA_{Ap}$ activity during food processing. Further research will be needed to know the physiological role of $TanA_{Ap}$ in *A. parvulum* metabolism.

The above results indicated that, even though *A. parvulum* tannase action could begin almost immediately after food ingestion, its contribution to tannin breakdown would not be relevant. Most of the tannin digestion could result from bacterial intestinal tannases rather than from oral tannase. In the microbiome of the major site of food tannin hydrolysis, the intestinal tract, at least three tannase-producing bacteria have been isolated, *L. plantarum*, *S. lugdunensis* or *S. gallolyticus* (Iwamoto et al., 2008; Noguchi et al., 2007; Rusniok et al., 2010; Sly, Cahill, Osawa, & Fujisawa, 1997). From these intestinal bacteria, only the biochemical properties of $TanB_{Lp}$ from *L. plantarum* have been studied. $TanB_{Lp}$ possesses adequate properties for intestinal tannin degradation. However, further testing would be required to define the metabolism of these phenolic compounds comprehensively. In particular, the activity of the complex communities of microorganisms present in all parts of the human digestive tract would need to be examined.

Conclusions

In the present study, a novel bacterial tannase namely $TanA_{Ap}$ from *A. parvulum*, an inhabitant of the human oral cavity, was purified. $TanA_{Ap}$ was biochemically characterized by using a sensitive colorimetric method. Among bacterial tannases, $TanA_{Ap}$ possessed low specific activity and was unable to hydrolyze complex tannins. These biochemical properties are not favorable for the breakdown of complex food tannins during oral processing.

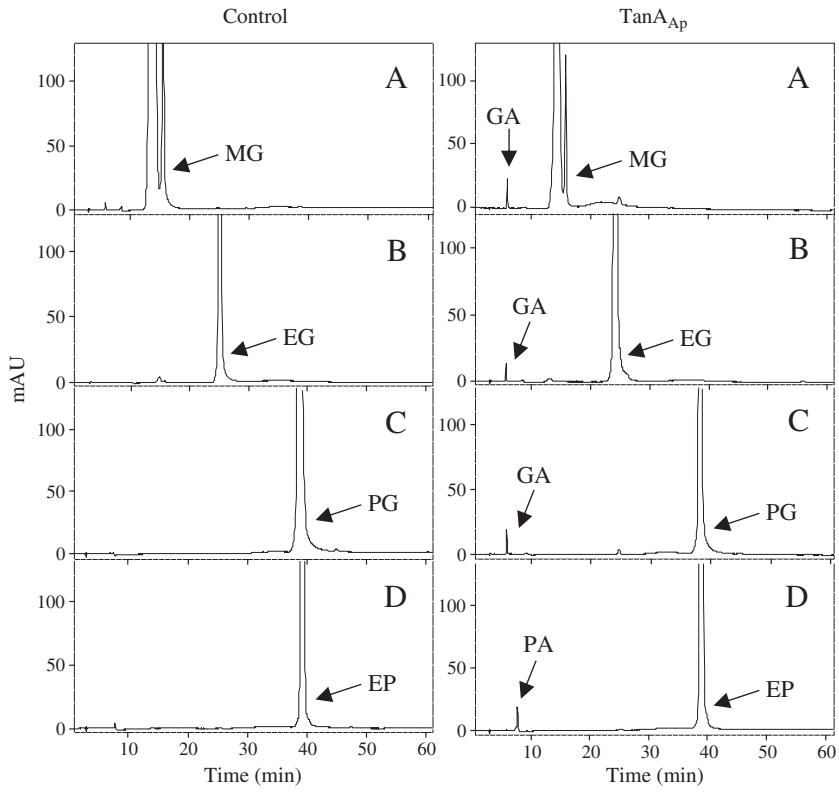

Fig. 3. Enzymatic activity of tannase from *A. parvulum* against gallic and protocatechuic acid esters. Hydrolase activity of purified TanA$_{Ap}$ compared with control reactions on which the enzyme was omitted. HPLC chromatograms of TanA$_{Ap}$ (200 μg) incubated in 50 mM phosphate buffer pH 6, and 1 mM of methyl gallate (A), ethyl gallate (B), propyl gallate (C), and ethyl protocatechuate (D). The methyl gallate (MG), ethyl gallate (EG), propyl gallate (PG), ethyl protocatechuate (EP), gallic acid (GA), and protocatechuic acid (PA) detected are indicated. The chromatograms were recorded at 280 nm.

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Capítulo 2

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Tannin Degradation by a Novel Tannase Enzyme Present in Some *Lactobacillus plantarum* Strains

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Lactobacillus plantarum **is frequently isolated from the fermentation of plant material where tannins are abundant. *L. plantarum* strains possess tannase activity to degrade plant tannins. An *L. plantarum* tannase ($TanB_{Lp}$, formerly called TanLp1) was previously identified and biochemically characterized. In this study, we report the identification and characterization of a novel tannase ($TanA_{Lp}$). While all 29 *L. plantarum* strains analyzed in the study possess the $tanB_{Lp}$ gene, the gene $tanA_{Lp}$ was present in only four strains. Upon methyl gallate exposure, the expression of $tanB_{Lp}$ was induced, whereas $tanA_{Lp}$ expression was not affected. $TanA_{Lp}$ showed only 27% sequence identity to $TanB_{Lp}$, but the residues involved in tannase activity are conserved. Optimum activity for $TanA_{Lp}$ was observed at 30°C and pH 6 in the presence of Ca^{2+} ions. $TanA_{Lp}$ was able to hydrolyze gallate and protocatechuate esters with a short aliphatic alcohol substituent. Moreover, $TanA_{Lp}$ was able to fully hydrolyze complex gallotannins, such as tannic acid. The presence of the extracellular $TanA_{Lp}$ tannase in some *L. plantarum* strains provides them an advantage for the initial degradation of complex tannins present in plant environments.**

Tannins are present in a variety of plants that are utilized as food and feed (1). Tannins seem to be a two-edged sword, since they are beneficial to health due to their chemopreventive activities against carcinogenesis and mutagenesis, but simultaneously, they may be involved in cancer formation, hepatotoxicity, or antinutritional activity (2). The molar mass of tannin molecules affects the tannin characteristics directly. It has been found that the higher the molar mass of tannin molecules, the stronger the antinutritional effects and the lower the biological activities (2). Small-molecule tannins are suggested to have fewer antinutritional effects and can be more readily absorbed.

Based on the molecular structure and origin of currently known tannins and their roles in plant life, tannins are defined as polyphenolic secondary metabolites of higher plants, and they are either galloyl esters or their derivatives, in which galloyl moieties or their derivatives are attached to a variety of polyol, catechin, and triterpenoid cores, or they are oligomeric and polymeric proanthocyanidins that can possess different interflavanyl coupling and substitution patterns (3). Gallotannins are those in which galloyl units or their metadepsidic derivatives are bound to diverse polyol, catechin, or triterpenoid units. Upon hydrolysis by acids, bases, or certain enzymes, gallotannins yield glucose and gallic acid (4).

Though tannins have toxic effects on various organisms, some microorganisms are resistant to tannins and have the ability to degrade them into oligomeric tannins and other useful derivatives, such as gallic acid or pyrogallol. Gallotannins are degraded by some bacteria, fungi, and yeasts, which can only hydrolyze the galloyl residues of galloyl esters of tannins. Tannin acyl hydrolase (EC 3.1.1.20), commonly known as tannase, catalyzes the hydrolysis of the galloyl ester bond of tannins. Tannase belongs to the superfamily of esterases. Since its discovery, tannase has found wide applications in the food, feed, beverage, pharmaceutical, and chemical industries (5). Despite the extensive interest and long history of the study of tannase, there is surprisingly little knowledge about the enzyme at the molecular level, which has become one of the critical factors that limit the large-scale application of tannase. To our knowledge, the only bacterial tannases that have been analyzed genetically are those from *Staphylococcus lugdunensis* (6), *Lactobacillus plantarum* (7, 8), and *Enterobacter* sp. (9). In addition, *L. plantarum* tannase has been biochemically and structurally characterized (7, 8, 10).

L. plantarum is a lactic acid bacterial species that is most frequently encountered in the fermentation of plant materials where tannins are abundant. These plant fermentations include several food and feed products, e.g., olives, grape must, and a variety of vegetable fermentation products. Among food lactic acid bacteria, strains from the *L. plantarum* group possess tannase activity (11–13). The biochemical pathway for the degradation of tannins by *L. plantarum* involves the action of a tannase and a gallate decarboxylase to decarboxylate the gallic acid formed by tannase action (14–16). The *L. plantarum* genes encoding tannase ($tanB_{Lp}$, formerly called *tanLp1*) (7) and gallate decarboxylase (*lpdBCD*) (16) involved in tannin degradation have been identified. However, an additional putative *L. plantarum* tannase sequence has been annotated in the genome of an *L. plantarum* strain. In this work, we have characterized the biochemical properties of this novel tannase. The presence of this tannase was analyzed among *L. plantarum* strains. Finally, the relative expression of both tannase genes under methyl gallate exposure was studied.

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MATERIALS AND METHODS

Strains and growth conditions. A total of 29 strains of *L. plantarum* were used in this study. *L. plantarum* strains WCFS1, NC8, and LPT 57/1 were kindly provided by M. Kleerebenzem (NIZO Food Research, The Netherlands), L. Axelsson (Norwegian Institute of Food, Fisheries and Aquaculture Research, Norway), and J. L. Ruíz-Barba (Instituto de la Grasa, CSIC; Spain), respectively. Seven strains were provided by the Spanish Type Culture Collection (CECT): *L. plantarum* CECT 220 (ATCC 8014), CECT 221 (ATCC 14431), CECT 223, CECT 224, CECT 749 (ATCC 10241), CECT 4645 (NCFB 1193), and the type strain *L. plantarum* subsp. *plantarum* CECT 748^T (ATCC 14917; DSMZ 20174). Seven strains were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ): *L. plantarum* DSM 1055, DSM 2648, DSM 10492, DSM 12028, DSM 13273, DSM 20246, and the type strain of *L. plantarum* subsp. *argentoratensis* DSM 16365^T. Eleven strains were isolated from grape must or wines of different wine-producing areas of Spain over the period from 1998 to 2001: *L. plantarum* RM28, RM31, RM34, RM35, RM38, RM39, RM40, RM41, RM71, RM72, and RM73) (17). The *L. plantarum* strains were routinely grown in de Man, Rogosa, and Sharpe medium (MRS) adjusted to pH 6.5 and incubated at 30°C. For the degradation assay, the *L. plantarum* strains were cultivated in a modified basal and defined medium described previously for *L. plantarum* (18). The basal medium was modified by the replacement of glucose by galactose. This defined medium was used to avoid the presence of phenolic compounds included in nondefined media. The sterilized modified basal medium was supplemented with filter-sterilized tannic acid (1 mM). The *L. plantarum*-inoculated medium was incubated in darkness without shaking at 30°C for 10 days. Incubated medium with cells and without phenolic compound and incubated medium without cells and with phenolic compounds were used as controls. The phenolic products were extracted from the supernatants twice with ethyl acetate (one-third of the reaction volume).

Escherichia coli DH10B was used for all DNA manipulations. *E. coli* BL21(DE3) was used for expression in the pURI3-TEV vector (19). *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37°C and 140 rpm. When required, ampicillin was added to the medium at a concentration of 100 μg/ml.

PCR detection of tannase-encoding genes. Genes encoding *L. plantarum* tannases (*tanA*$_{Lp}$ and *tanB*$_{Lp}$) were amplified by PCR using chromosomal DNA from several lactic acid bacterial strains. The *tanB*$_{Lp}$ gene (1.4 kb) was amplified by using primers 951 (5′-TGATGCTGACTGGCT GGTGC) and 952 (5′-GCACAAGCCATCAATCCAGG). Oligonucleotides 953 (5′-CCTGATGAGTGGTTTGTTAG) and 954 (5′-CTTGCGTT CTGCTTCGGTATG) were used to amplify the *tanA*$_{Lp}$ gene (1.8 kb). The reactions were performed in a Mastercycler personal thermal cycler (Eppendorf), using 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 30 s. The amplified fragments were resolved in agarose gels.

RNA isolation, real-time (RT)-PCR, and qPCR. For RNA isolation, *L. plantarum* MRS cultures were grown to an optical density at 600 nm (OD_{600}) of 1 and then supplemented with methyl gallate at 15 mM final concentration. As a control, RNA was also isolated from cultures not supplemented with methyl gallate. After 10 min of incubation, the cultures were immediately processed for RNA extraction as previously described (20). After DNase I treatment, the absence of DNA from the RNA samples was verified by PCR. The DNA-free RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. From the DNA obtained, quantitative gene expression was analyzed in an AbiPrism 7500 Fast Real Time PCR system (Applied Biosystems). Specific primer pairs were designed with the Primer Express 3.0 program to amplify internal regions of tannase genes. Oligonucleotides 1168 (5′-TGCGCTACCGTG GGATATTC) and 1169 (5′-AATCCAGGAAAATAAATCGCCTAA) were used to amplify 64 bp of *tanB*$_{Lp}$, and primers 1286 (5′-AAAGCAAGCTA CGCCAAAGC) and 1287 (5′-CCCTGGGCATCCGTCTTC) were used to amplify a 56-bp fragment of *tanA*$_{Lp}$. The expression level of the endogenous control gene (*ldh*) was assayed with primers 918 (5′-AACCGCGAC AATGTTTTGATT) and 919 (5′-TTGTGAACGGCAGTTTCAGTGT). Amplifications were performed in triplicate. All quantitative-PCR (qPCR) assays amplified a single product, as determined by melting-curve analysis and by electrophoresis. A standard curve was plotted with cycle threshold (C_t) values obtained from amplification of known quantities of cDNA and used to determine the efficiency (E) as follows: $E = 10^{-1/\text{slope}}$. In order to measure *L. plantarum* gene expression, amplification of the endogenous control gene was performed simultaneously, and its relative expression was compared with that of the target gene. Relative expression levels were calculated with the Applied Biosystems 7500 Fast System relative quantification software using the *L. plantarum ldh* gene as the endogenous gene and growth in the absence of methyl gallate as the growth condition calibrator.

Expression and purification of TanA$_{Lp}$ from *L. plantarum* ATCC 14917^T. As a peptide signal was predicted in the TanA$_{Lp}$ sequence, the gene *tanA*$_{Lp}$ in the locus HPREF0531_11477 from *L. plantarum* ATCC 14917^T was PCR amplified, but lacking the 22-amino-acid peptide signal. The gene was amplified with Prime Star HS DNA polymerase (TaKaRa) by using the primers 805 (5′-*GGTGAAAACCTGTATTTCCAGGGC*gcttg cggacactccgaaacgaaga) and 637 (5′-*ATCGATAAGCTTAGTTAGCTAT*ta cttcaagctcttgttgacccactta) (the nucleotides pairing with the expression vector sequence are in italics, and the nucleotides pairing with the *tanA*$_{Lp}$ gene sequence are in lowercase). The gene was cloned into the pURI3-TEV vector, which encodes expression of a leader sequence containing a six-histidine affinity tag. The purified PCR product was then inserted into the pURI3-TEV vector by using a restriction enzyme- and ligation-free cloning strategy (19). *E. coli* DH10B cells were transformed, and the recombinant plasmids were isolated. Those containing the correct insert were used for transformation of *E. coli* BL21(DE3) cells.

E. coli cells carrying the recombinant pURI3-TEV-TanA$_{Lp}$ plasmid were grown at 37°C in LB medium containing ampicillin (100 μg/ml) and induced by adding 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After induction, the cells were grown at 22°C for 20 h and harvested by centrifugation (7,500 × *g* for 15 min at 4°C). The cells were resuspended in 50 mM sodium phosphate buffer, pH 7.0, containing 300 mM NaCl. Crude extracts were prepared by French press lysis of cell suspensions (three cycles at 1,100 lb/in^2). The lysates were centrifuged at 17,400 × *g* for 40 min at 4°C. The supernatant obtained was filtered through a 0.22-μm filter (Millipore) and gently mixed for 20 min at room temperature with 1 ml Talon resin (Clontech). The resin was washed with 50 mM sodium phosphate buffer, pH 7.0, containing 300 mM NaCl and 10 mM imidazole. The recombinant His$_6$-tagged protein was eluted with 50 mM sodium phosphate, pH 7.0, containing 300 mM NaCl and 150 mM imidazole. The eluted His$_6$-tagged TanA$_{Lp}$ was dialyzed overnight at 4°C against 50 mM sodium phosphate buffer, pH 7.0, containing 300 mM NaCl. The purity of the enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in Tris-glycine buffer.

Tannase activity assay. Since tannase catalyzes the hydrolysis of the galloyl ester linkage liberating gallic acid, the activity of tannase could be measured by estimating the gallic acid formed due to enzyme action (21). A method specific for the detection of gallic acid could be used for a reliable quantification of tannase activity. Inoue and Hagerman described a rhodanine assay for determining free gallic acid (22). Rhodanine reacts only with gallic acid and not with galloyl esters of other phenolics. Rhodanine reacts with the vicinal hydroxyl groups of gallic acid to give a red complex with maximum absorbance at 520 nm. Since the rhodanine assay using commercial tannic acid as the substrate gives high absorbance values due to small amounts of free gallic acid present in the preparation, methyl gallate was used as the substrate.

Gallic acid analysis in reactions was determined in triplicate by using the following assay. TanA$_{Lp}$ (10 μg) in 700 μl of 50 mM phosphate buffer, pH 6.5, was incubated with 40 μl of 25 mM methyl gallate (1 mM final concentration) for 5 min at 37°C. After incubation, 150 μl of a methanolic

rhodanine solution (0.667% rhodanine in 100% methanol) was added to the mixture. After 5 min of incubation at 30°C, 100 μl of 0.5 M KOH was added. The absorbance at 520 nm was measured on a spectrophotometer. A standard curve using gallic acid concentrations ranging from 0.125 to 1 mM was prepared. One unit of tannase activity was defined as the amount of enzyme required to release 1 μmol of gallic acid per minute under standard reaction conditions.

Biochemical characterization of $TanA_{Lp}$. Activities of $TanA_{Lp}$ from *L. plantarum* ATCC 14917^T were measured at 4, 20, 30, 37, 45, 55, and 65°C to determine the optimal temperature for enzymatic activity. The optimum pH value for tannase activity was determined by studying its pH dependence within the pH range between 3 and 10. Acetic acid-sodium acetate buffer was used for pH 3 to 5, citric acid-sodium citrate buffer for pH 6, sodium phosphate buffer for pH 7, Tris-HCl buffer for pH 8, glycine-NaOH buffer for pH 9, and sodium carbonate-bicarbonate for pH 10. A 100 mM concentration was used in all the buffers. The rhodanine assay was used for the optimal pH characterization of $TanA_{Lp}$. Since the rhodanine-gallic acid complex forms only under basic conditions, after the enzymatic degradation of methyl gallate, KOH was added to the reaction mixture to ensure that the same pH value (pH 11) was achieved in all samples assayed.

For temperature stability measurements, $TanA_{Lp}$ was incubated in 50 mM phosphate buffer, pH 6.5, at 22, 30, 37, 45, 55, and 65°C for 15 min, 30 min, and 1, 2, 5, and 18 h. After incubation, the residual activity was measured as described above.

To test the effects of metals and ions on the activity of $TanA_{Lp}$, the enzymatic activity was measured in the presence of different additives at a final concentration of 1 mM. The additives analyzed were $MgCl_2$, KCl, $CaCl_2$, $HgCl_2$, $ZnCl_2$, Triton X-100, urea, Tween 80, EDTA, dimethyl sulfoxide (DMSO), and β-mercaptoethanol. All the determinations were done in triplicate.

$TanA_{Lp}$ substrate specificity analysis by HPLC. The activity of $TanA_{Lp}$ against 21 potential substrates was analyzed. The substrates assayed were gallic esters (methyl gallate, ethyl gallate, propyl gallate, and lauryl gallate), benzoic esters (methyl benzoate and ethyl benzoate), hydroxybenzoic esters (methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate, and butyl 4-hydroxybenzoate), vanillic ester (methyl vanillate), dyhydroxybenzoic esters (methyl 2,4-dihydroxybenzoate, ethyl 3,4-dihydroxybenzoate or protocatechuic acid ethyl ester, and ethyl 3,5-dihydroxybenzoate), gentisic ester (methyl gentisate), salicylic ester (methyl salicylate), and ferulic esters (ferulic methyl ester and ferulic ethyl ester). Tannic acid and epigallocatechin gallate were also assayed as potential substrates. Tannase (50 μg), in phosphate buffer, pH 6.0 (50 mM), and $CaCl_2$ (1 mM), was incubated at 37°C in the presence of the substrate (1 mM). As controls, phosphate buffers containing the reagents but not the enzyme were incubated under the same conditions. The reaction products were extracted twice with ethyl acetate; the solvent fractions were filtered through a 0.45-μm polyvinylidene difluoride (PVDF) filter and analyzed by high-performance liquid chromatography with diode array detection (HPLC-DAD). A Thermo chromatograph (Thermo Electron Corporation, Waltham, MA, USA) equipped with a P4000 SpectraSystem pump, an AS3000 autosampler, and a UV6000LP photodiode array detector were used. A gradient of solvent A (water-acetic acid, 98:2 [vol/vol]) and solvent B (water-acetonitrile-acetic acid, 78:20:2 [vol/vol/vol]) was applied to a reversed-phase Nova-pack C_{18} cartridge (25 cm by 4.0-mm inside diameter [i.d.]; 4.6-μm particle size) at room temperature as follows: 0 to 55 min, 80% B linear, 1.1 ml/min; 55 to 57 min, 90% B linear, 1.2 ml/min; 57 to 70 min, 90% B isocratic, 1.2 ml/min; 70 to 80 min, 95% B linear, 1.2 ml/min; 80 to 90 min, 100% linear, 1.2 ml/min; 100 to 120 min, washing, 1.0 ml/min; and reequilibration of the column under the initial gradient conditions. Samples were injected onto the cartridge after being filtered through a 0.45-μm PVDF filter. Detection of the substrates and the degradation compounds was performed spectrophotometrically by scanning from 220 to 380 nm. The identification of degradation compounds was carried out by comparing the retention times and spectral data of each peak with those of standards from commercial suppliers or by LC-DAD–electrospray ionization-mass spectrometry (ESI-MS).

Sequence data analysis. A homology search with finished and unfinished microbial genome databases was performed with the BLAST algorithm at the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). Multiple alignments were made using the Clustal W2 Program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) on the EBI site after retrieval of sequences from the GenBank and Swiss-Prot databases. Computer promoter predictions were carried out at the Internet site (http://www.fruitfly.org/seq_tools/promoter.html). pI and molecular weight (MW) were analyzed on EXPASY (http://web.expasy.org/compute_pi/), signal peptide cleavage sites were analyzed on the SignalP 4.1 server site (http://www.cbs.dtu.dk/services/SignalP/), and predicted transcription terminators were analyzed at the ARNold site (http://rna.igmors.u-psud.fr/toolbox/arnold/index.php#Results).

RESULTS

Presence of a putative novel tannase-encoding gene in several *L. plantarum* strains. Homology searches in genome databases allowed us to find the locus HMPREF0531_11477 in the *L. plantarum* ATCC 14917^T genome, which encodes a 626-amino-acid-residue protein annotated as "tannase," defined here as $TanA_{Lp}$. Interestingly, multiple amino acid sequence alignments of $TanA_{Lp}$ from *L. plantarum* ATCC 14917^T with $TanA_{Sl}$ tannase from *S. lugdunensis* and $TanB_{Lp}$ tannase from *L. plantarum* revealed high sequence identity to $TanA_{Sl}$ (50%) and much lower identity to $TanB_{Lp}$ (27%) (see Fig. S1 in the supplemental material). Moreover, $TanA_{Lp}$ shares additional features with $TanA_{Sl}$ from *S. lugdunensis*. Both are 67-kDa proteins and have alkaline isoelectric points (9.54 for $TanA_{Sl}$ and 9.94 for $TanA_{Lp}$), and they present predicted signal peptides. On the other hand, $TanB_{Lp}$ is a 50-kDa protein with an isoelectric point of 6.06, and it does not possess a signal peptide.

Based on the recently described crystal structure of $TanB_{Lp}$ (10), the conserved sequence motif Gly-X-Ser-X-Gly typical of serine hydrolases could be easily identified in $TanA_{Lp}$ from *L. plantarum* ATCC 14917^T (Gly-215 to Gly-219 in $TanA_{Lp}$). From the catalytic triad identified in the structure (Ser-163, His-451, and Asp-419 in $TanB_{Lp}$), only serine and histidine residues are conserved in $TanA_{Lp}$ as well as in $TanA_{Sl}$ (see Fig. S1 in the supplemental material), with Asp-419 being replaced by a Gln residue in both proteins. On the other hand, the residues which make contacts with the three hydroxyl groups of gallic acid (Asp-421, Lys-343, and Glu-357 in $TanB_{Lp}$) are conserved in both $TanA_{Lp}$ and $TanA_{Sl}$ (see Fig. S1 in the supplemental material).

The genomes of 10 *L. plantarum* strains are currently available. Analyses of these genomes revealed that a copy of the *$tanA_{Lp}$* gene is present only in *L. plantarum* ATCC 14917^T and *L. plantarum* NC8 and is not found in the rest (*L. plantarum* WCFS1, JDM1, ST-III, 16, P-8, IPLA 88, UCMA 3037, and ZJ316). In the ATCC 14917^T and NC8 strains, *$tanA_{Lp}$* is located between the genes *nox3* (encoding a NADH oxidase [accession number EFK29315]) and *dapE1* (encoding succinyl-diaminopimelate desuccinylase [accession number EFK29313]) (Fig. 1). The intergenic region between these genes is 121 bp long in the *L. plantarum* strains devoid of *$tanA_{Lp}$*; however, in the strains possessing *$tanA_{Lp}$*, this region is 2,515 or 2,516 bp long in NC8 and ATCC 14917^T, respectively. In *L. plantarum* ATCC 14917^T, this region encodes a 626-amino-acid-residue protein ($TanA_{Lp}$), which is preceded by a putative

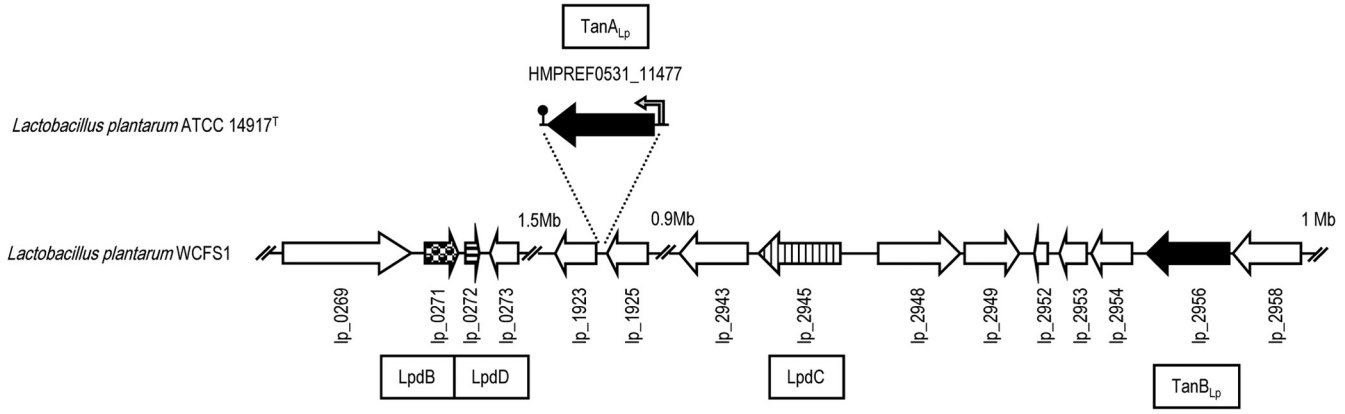

FIG 1 Genetic organization of the *L. plantarum* WCFS1 chromosomal region containing the gallate decarboxylase- and tannase-encoding genes (accession no. NC_004567; positions 243093 to 252815, 1743368 to 1746325, and 2618290 to 2635122). The insertion of the *tanA$_{Lp}$* gene in *L. plantarum* ATCC 14917^T is also represented (accession no. ACGZ02000013.1; positions c/26013 to 27893). The arrows represent genes. Genes coding for putative tannase proteins are represented by black arrows. Genes encoding gallate decarboxylase subunits (Lpd, LpdC, and LpdD) are also shown. The locations of a putative *tanA$_{Lp}$* promoter (vertical bent arrow) and transcription terminator region (ball and stick) are also indicated.

promoter, as revealed by sequence analysis. In turn, a putative transcription terminator site follows the stop codon, where a possible stem-loop structure is predicted, which would start 45 nucleotides downstream from the TAA stop codon and would have a 19-base stem and a 22-base loop. This structure may serve as a terminator for transcription (Fig. 1). The *L. plantarum* NC8 sequence is identical to that of ATCC 14917^T with the exception of a G deletion, which produces a frameshift from Gln-529 to the end of TanA$_{Lp}$ (see Fig. S2 in the supplemental material).

In order to know the extent of the tannase genes among *L. plantarum* strains, the presence of the *tanB$_{Lp}$* and *tanA$_{Lp}$* genes was studied in 29 *L. plantarum* strains isolated from different sources. To determine the presence of both genes, chromosomal DNA was extracted and PCR amplified. DNA fragments of 1.4 or 1.8 kb from *tanB$_{Lp}$* or *tanA$_{Lp}$*, respectively, were PCR amplified using oligonucleotides designed on the basis of the *L. plantarum* ATCC 14917^T sequence. All the *L. plantarum* strains analyzed gave the corresponding *tanB$_{Lp}$* amplicon (data not shown), which indicates that TanB$_{Lp}$ is generally present among *L. plantarum* strains, as described previously (7). Apart from ATCC 14917^T and NC8, two additional strains also possessed a copy of the *tanA$_{Lp}$* gene, namely, *L. plantarum* CECT 749 and RM35 (data not shown). Since the NC8 *tanA$_{Lp}$* copy is truncated, the complete sequences of the CECT 749 and RM35 strains were determined. Alignment of the resulting TanA$_{Lp}$ amino acid sequences revealed one substitution in the CECT 749 protein (Ala-107 to Asp-107) and two in the RM35 strain (Arg-563 to Lys-563, and Arg-565 to Gln-565) (see Fig. S2 in the supplemental material). It is noteworthy that these two mutated arginine residues are part of a motif (WRIR) conserved in all tannases (see Fig. S1 in the supplemental material).

Extracellular tannase activity on *L. plantarum* strains. Since the sequences of the four *tanA$_{Lp}$* copies present in the *L. plantarum* strains analyzed here differ, an activity assay was done to determine the functionality of the resulting coded proteins. In this regard, the presence of a putative signal peptide indicated that TanA$_{Lp}$ could be an extracellular protein, and in fact, an extracellular tannase produced by an *L. plantarum* strain has been reported (23). *L. plantarum* WCFS1 and the four strains possessing a *tanA$_{Lp}$* copy were grown in a basal medium containing 1 mM tannic acid for 10 days. Tannic acid was chosen because it is a complex gallotannin unable to pass into the cell to be degraded by intracellular TanB$_{Lp}$ tannase. As a control, the medium was incubated under the same conditions. From the *L. plantarum* culture, the cells were pelleted, and the tannic acid in the supernatant was extracted and analyzed by HPLC. Figure 2 shows that most of the chromatograms were similar to the control, and only small variations were observed among them. However, *L. plantarum* ATCC 14917^T showed a chromatogram that clearly indicated hydrolysis of the high-molecular-weight tannins, suggesting the presence of an active extracellular tannase only in this strain.

Relative expression of *L. plantarum* tannase genes under methyl gallate exposure. As tannase is involved in tannin degradation, the relative expression of both tannase-encoding genes under methyl gallate exposure was studied. Strain WCFS1 was analyzed as a model strain with only one tannase enzyme and strain ATCC 14917^T as a model strain having two different active tannase proteins. *L. plantarum* cultures were induced for 10 min by the presence of 15 mM methyl gallate as a potential tannase substrate. The gene expression levels obtained were substantially different between the two tannase-encoding genes (data not shown), indicating the presence of two different expression patterns for the proteins. The *tanA$_{Lp}$* gene, only present in *L. plantarum* ATCC 14917^T, showed an expression level not affected by the presence of its substrate, methyl gallate. However, the *tanB$_{Lp}$* gene expression profiles were affected. In both *L. plantarum* strains, the presence of 15 mM methyl gallate induces about a 3-fold increase in the expression of the *tanB$_{Lp}$* gene.

Biochemical properties of TanA$_{Lp}$ from *L. plantarum* ATCC 14917^T. Of the *L. plantarum* strains possessing two tannase enzymes, only *L. plantarum* ATCC 14917^T showed extracellular tannase activity; therefore, TanA$_{Lp}$ from this strain was biochemically characterized. The *tanA$_{Lp}$* gene lacking the 22-amino-acid peptide signal sequence from *L. plantarum* ATCC 14917^T was expressed in *E. coli* under the control of an inducible promoter. Cell extracts were used to detect the presence of overproduced proteins by SDS-PAGE analysis. Control cells containing the pURI3-TEV vector plasmid did not show protein overexpression; in addition, no tannase activity was observed in this control extract. However, an

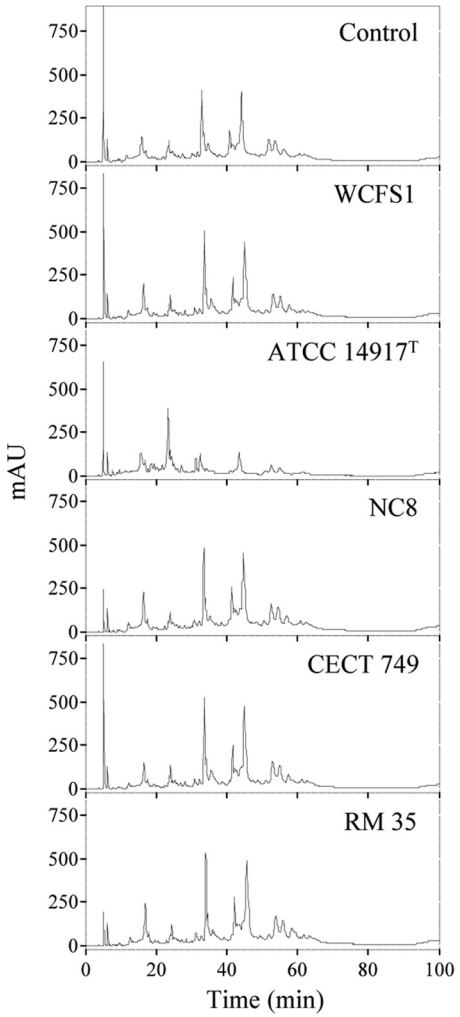

FIG 2 HPLC analysis of tannic acid degradation by *L. plantarum* cultures. Modified basal medium containing 1 mM tannic acid was inoculated with *L. plantarum* strains (WCFS1, ATCC 14917^T, NC8, CECT 749, and RM 35) and incubated for 10 days. A noninoculated control medium was incubated under the same conditions. Detection was performed at 280 nm. AU, absorbance units.

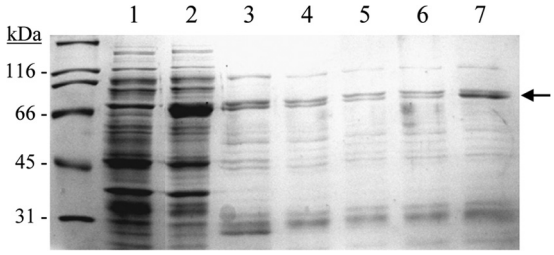

FIG 3 Purification of *L. plantarum* $TanA_{Lp}$ protein. Shown are SDS-PAGE analysis of the expression and purification of His_6-$TanA_{Lp}$ and analysis of soluble cell extracts of IPTG-induced *E. coli* BL21(DE3)(pURI3-TEV) (lane 1) and *E. coli* BL21(DE3)(pURI3-TEV-$TanA_{Lp}$) (lane 2) or fractions eluted after His affinity resin (lanes 3 to 7). The arrow indicates the overproduced and purified protein. The 12.5% gel was stained with Coomassie blue. Molecular mass markers are located on the left (SDS-PAGE Standards; Bio-Rad).

overproduced protein with an apparent molecular mass of around 67 kDa was observed in cells harboring pURI3-TEV-$TanA_{Lp}$ (Fig. 3). Since the cloning strategy yielded a His-tagged protein variant, *L. plantarum* pURI3-TEV-$TanA_{Lp}$ could be purified on an immobilized metal affinity chromatography (IMAC) resin. However, unexpectedly, the protein was scarcely purified (2.71 mg/liter), with most of the protein not being bound to the resin.

The *L. plantarum* ATCC 14917^T $TanA_{Lp}$ enzyme, partially purified by the affinity resin, was biochemically characterized. Tannase activity was determined by using methyl gallate as the substrate. The specific activities of $TanA_{Lp}$ and $TanB_{Lp}$ (taken as references) were determined by the rhodanine assay. $TanA_{Lp}$ has a specific activity of 39 U/mg, whereas $TanB_{Lp}$ has 404 U/mg.

Figure 4 shows the optimum temperature and pH and the thermal stability of $TanA_{Lp}$ as determined by the rhodanine assay, with methyl gallate as the substrate. $TanA_{Lp}$ displays optimal activity within the 20 to 30°C temperature range and an optimal pH around 6. Figure 4D shows the effects of various additives (1 mM final concentration) on the enzymatic activity of $TanA_{Lp}$. $CaCl_2$ greatly increased and $HgCl_2$ completely inhibited $TanA_{Lp}$ activity. In relation to $TanA_{Lp}$ substrates, similarly to $TanB_{Lp}$, only the esters derived from gallic and protocatechuic acids were hydrolyzed (see Fig. S3 in the supplemental material). It seems that other cinnamic acids without hydroxyl groups and with substituents other than —H or —OH at position 2 were not metabolized by $TanA_{Lp}$ or by $TanB_{Lp}$. Regarding the aliphatic alcohol constituent of the ester bond, a lauryl substituent could not be effectively hydrolyzed by $TanA_{Lp}$ (see Fig. S3 in the supplemental material).

DISCUSSION

Although tannase activity has been extensively described in *L. plantarum* strains (12–14), the first bacterial tannase genetically identified was $TanA_{Sl}$ from *S. lugdunensis* (6), and then, by sequence comparison, $TanB_{Lp}$ (Lp_2956) from *L. plantarum* was later identified (7). The first complete genome of an *L. plantarum* strain was from the WCFS1 strain. However, at present, the sequences of several *L. plantarum* strains are publicly available. In *L. plantarum* ATCC 14917^T, the locus HMPREF0531_11477 was annotated as tannase and designated $TanA_{Lp}$. $TanA_{Lp}$ from *L. plantarum* ATCC 14917^T showed higher sequence identity to $TanA_{Sl}$ from *S. lugdunensis* (50%) than to *L. plantarum* $TanB_{Lp}$ tannase (27%). Despite the low identity to $TanB_{Lp}$, $TanA_{Sl}$ and $TanA_{Lp}$ shared $TanB_{Lp}$ motifs involved in tannase activity (10). Therefore, structural data also suggest that $TanA_{Lp}$ from *L. plantarum* ATCC 14917^T could be an active tannase.

When the presence of both tannases was studied in 36 *L. plantarum* strains, it was observed that a copy of the *tanB*$_{Lp}$ gene was present in all the analyzed strains, whereas *tanA*$_{Lp}$ was present in only four *L. plantarum* strains (ATCC 14917^T, NC8, CECT 749, and RM35). The analysis of the $TanA_{Lp}$ protein sequences from these four strains revealed that, compared to $TanA_{Lp}$ from *L. plantarum* ATCC 14917^T, $TanA_{Lp}$ from CECT 749 and RM35 possessed amino acid substitutions in a motif conserved in all tannases. Moreover, the sequence of *tanA*$_{Lp}$ from *L. plantarum* NC8 posses a frameshift that produces a truncated protein lacking 2 of the 3 residues of the catalytic triad (residues equivalent to His-451 and Asp-419 in $TanB_{Lp}$) and Asp-421, which make contacts with a hydroxyl group of gallic acid.

From the sequence analysis, $TanA_{Lp}$ from *L. plantarum* ATCC 14917^T seems to be the only $TanA_{Lp}$ tannase in which the residues important for activity are conserved. In order to verify this hy-

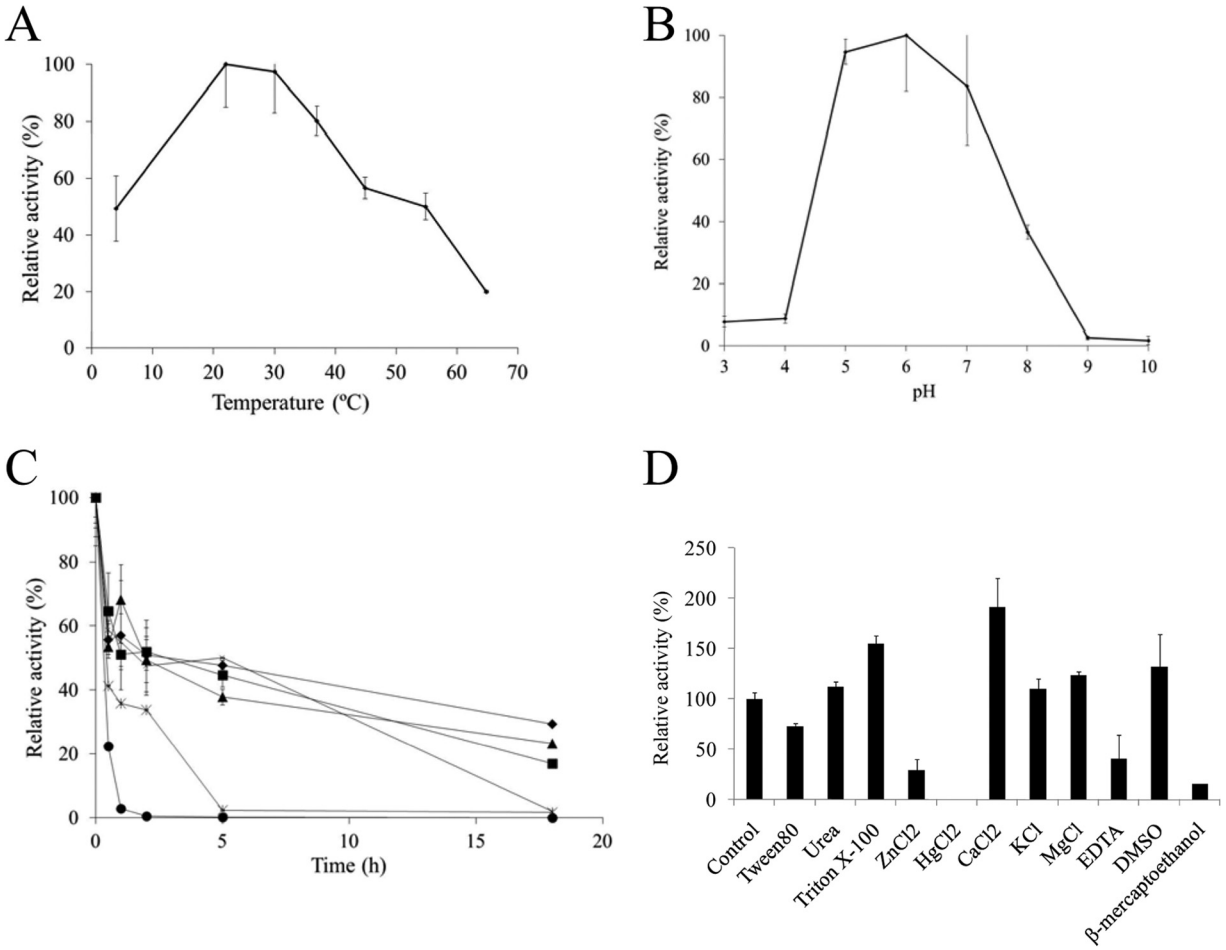

FIG 4 Some biochemical properties of $TanA_{Lp}$ protein. (A) Relative activity of $TanA_{Lp}$ versus temperature. (B) Relative activity versus pH. (C) Thermal stability of $TanA_{Lp}$ after preincubation at 22°C (diamonds), 30°C (squares), 37°C (triangles), 45°C (×), 55°C (stars), and 65°C (circles) in phosphate buffer (50 mM; pH 6.5). At the indicated times, aliquots were withdrawn and analyzed as described in Materials and Methods. The experiments were done in triplicate. The mean values and standard errors are shown. The observed maximum activity was defined as 100%. (D) Relative activity of $TanA_{Lp}$ after incubation with 1 mM concentrations of different additives. The activity of the enzyme incubated in the absence of additives was defined as 100%.

pothesis, and taking into account that $TanA_{Lp}$ seems to be an extracellular tannase, strains possessing a *tanA*$_{Lp}$ copy (ATCC 14917^T, NC8, CECT 749, and RM35) were grown in the presence of tannic acid, a complex gallotannin unable to pass into the cell to be degraded by intracellular $TanB_{Lp}$ tannase. As expected, the presence of an active extracellular tannase was observed only in *L. plantarum* ATCC 14917^T. In this strain, in addition to the functionality of $TanA_{Lp}$, the functionality of $TanB_{Lp}$ was previously demonstrated (8). As the strain possesses two tannase proteins able to hydrolyze gallotannins, the expression of these proteins was studied under methyl gallate exposure. The presence of the substrate methyl gallate did not affect the expression of *tanA*$_{Lp}$; however, it increased the expression of *tanB*$_{Lp}$. This expression behavior allows the assumption that *tanB*$_{Lp}$ encodes an inducible tannase in *L. plantarum* ATCC 14917^T, as previously observed in the WCFS1 strain under tannic acid challenge (24).

From the results obtained in this study, it seems that the two *L. plantarum* tannases play different physiological roles. These different functions could be partially attributed to their different biochemical properties, such as their reported substrate spectra, which seem to be very similar except that esters having a long aliphatic alcohol were not effectively hydrolyzed by $TanA_{Lp}$. Moreover, $TanA_{Lp}$ from *L. plantarum* ATCC 14917^T has a specific activity of 39 U/mg, 10 times lower than the specific activity calculated for $TanB_{Lp}$ (404 U/mg) from the same strain. In addition, the optimal temperature and pH for $TanA_{Lp}$ (20 to 30°C and pH 6) differed from those described for $TanB_{Lp}$ (40°C and pH 7 to 8) (7, 8). Interestingly, the optimal activity of $TanA_{Lp}$ is similar to the optimal tannase activity shown by cell extracts from *L. plantarum* ATCC 14917^T (25). It is noteworthy that *L. plantarum* ATCC 14917^T cell extracts were obtained from cultures grown in a medium devoid of possible tannase inducers, on which $TanB_{Lp}$ tannase could not be induced. Therefore, it seems that in the absence of a substrate, the biochemical characteristics of tannase activity shown by cell extracts of *L. plantarum* ATCC 14917^T, containing both tannase genes, are more similar to those exhibited by $TanA_{Lp}$ than to those of $TanB_{Lp}$. This could indicate that, in the absence of a substrate, $TanA_{Lp}$ activity predominates in *L. plantarum* strains having two tannase enzymes. The gene expression results indicated that $TanA_{Lp}$ is not inducible by the presence of methyl gallate; however, its basal expression level could be enough to be detected in *L. plantarum* cell extracts. In complex tannins, such as

tannic acid, the presence of an extracellular and low-activity $TanA_{Lp}$ tannase in some *L. plantarum* strains could provide an enzymatic activity able to partially degrade tannic acid outside the cell. The less complex tannins originated by $TanA_{Lp}$ action could be able to induce the expression of $tanB_{Lp}$ and pass into the cell to be degraded by $TanB_{Lp}$. In addition, $TanA_{Lp}$ biochemical properties are more convenient than those from $TanB_{Lp}$ for an extracellular enzyme acting on plant substrates. Temperatures around 20 to 30°C and acidic pH could be environmental conditions present on these plant substrates.

The specific catabolic capacity of *L. plantarum* against gallotannins suggests that they provide a selective advantage to the species for life in environments where compounds of plant origin are abundant. The presence in some *L. plantarum* strains of a second active tannase provides an additional advantage. *L. plantarum* should be able to degrade them with $TanA_{Lp}$ and does not depend on other microorganisms for the initial degradation of these compounds. This second tannase, $TanA_{Lp}$, is an extracellular enzyme able to hydrolyze complex gallotannins, which are unable to pass into the cell to be degraded by $TanB_{Lp}$. Moreover, the presence of $TanA_{Lp}$ provides *L. plantarum* with strains an additional response mechanism to overcome the adverse effects of tannins present in their environment.

ACKNOWLEDGMENTS

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Capítulo 3

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Uncovering the *Lactobacillus plantarum* WCFS1 Gallate Decarboxylase Involved in Tannin Degradation

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Lactobacillus plantarum **is a lactic acid bacterium able to degrade tannins by the subsequent action of tannase and gallate decarboxylase enzymes. The gene encoding tannase had previously been identified, whereas the gene encoding gallate decarboxylase is unknown. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of gallic-acid induced *L. plantarum* extracts showed a 54-kDa protein which was absent in the uninduced cells. This protein was identified as Lp_2945, putatively annotated UbiD. Homology searches identified *ubiD*-like genes located within three-gene operons which encoded the three subunits of nonoxidative aromatic acid decarboxylases. *L. plantarum* is the only bacterium in which the *lpdC* (*lp_2945*) gene and the *lpdB* and *lpdD* (*lp_0271* and *lp_0272*) genes are separated in the chromosome. Combination of extracts from recombinant *Escherichia coli* cells expressing the *lpdB*, *lpdC*, and *lpdC* genes demonstrated that LpdC is the only protein required to yield gallate decarboxylase activity. However, the disruption of these genes in *L. plantarum* revealed that the *lpdB* and *lpdC* gene products are essential for gallate decarboxylase activity. Similar to *L. plantarum* tannase, which exhibited activity only in esters derived from gallic and protocatechuic acids, purified His6-LpdC protein from *E. coli* showed decarboxylase activity against gallic and protocatechuic acids. In contrast to the tannase activity, gallate decarboxylase activity is widely present among lactic acid bacteria. This study constitutes the first genetic characterization of a gallate decarboxylase enzyme and provides new insights into the role of the different subunits of bacterial nonoxidative aromatic acid decarboxylases.**

Vegetable tannins are present in a variety of plants utilized as food and feed. High tannin concentrations are found in nearly every part of the plant, such as the bark, wood, leaf, fruit, root, and seed. Tannins also widely occur in common foodstuffs, such as pomegranate, banana, strawberry, grape, cashew nut, and hazelnut. Drinks like wine and tea also contain these phenolic compounds (1). Tannins have been described to exhibit opposing health effects (2). They are beneficial to health due to their chemopreventive activities against carcinogenesis and mutagenesis. However, tannins are considered nutritionally undesirable because of their ability to bind to proteins to form indigestible complexes and to chelate heavy metals, and they may be involved in cancer formation and hepatotoxicity (2).

Tannase (tannin acyl hydrolase, EC 3.1.1.20) catalyzes the hydrolysis of ester linkages in hydrolyzable tannins. The products of hydrolysis are glucose and gallic acid. In addition to esters, gallic acid can be found in plants in the free state or in the form of ethers (e.g., syringic acid and other lignins constituents), being a major pollutant present in the wastewater generated in processes involving the boiling of cork and in food manufacturing industries. Gallic acid and its derivatives are used in industry as antioxidants (3).

Although gallic acid is widely distributed in nature, it is easily oxidized at neutral or alkaline pH, at which point it becomes a product difficult for bacteria to use as a carbon source for growth. In fact, only bacteria of the genus *Pseudomonas* have been reported to be able to utilize free gallic acid as the sole carbon and energy source under aerobic conditions (4). The aerobic metabolism of gallic acid usually starts with a direct ring-cleavage reaction and formation of the central intermediate 4-oxalomesaconic acid, which then undergoes hydration to 4-carboxy-4-hydroxy-2-oxoadipic acid and aldol cleavage to oxaloacetic and pyruvic acids (5). In addition to microorganisms that use gallic acid as the sole carbon and energy source, there are also microorganisms that nonoxidatively decarboxylate gallic acid but do not possess appropriate mechanisms to further degrade the pyrogallol produced by this dead-end pathway. Strains of the species *Pantoea agglomerans* (6), *Enterococcus faecalis* (7), *Klebsiella pneumoniae* (7), *Streptococcus gallolyticus* (8), and *Lactobacillus plantarum* (9–11) were described to decarboxylate gallic acid to pyrogallol, without further metabolism. Even though several gallate decarboxylases, mainly from anaerobic sources, have been described, most of these enzymes have not been purified due to their instability. Gallate decarboxylases from *E. faecalis* and *P. agglomerans* are inducible enzymes which, due to their oxygen sensitivity, were extremely unstable when they were purified (6, 7). So far, none of these gallate decarboxylase enzymes has been genetically identified or characterized.

L. plantarum is a lactic acid bacterial species that is most frequently encountered in the fermentation of plant materials where tannins are abundant. These plant fermentations include several food and feed products, e.g., olives, grape must, and a variety of vegetable fermentation products. Among food lactic acid bacteria, strains from the *L. plantarum* group are the only ones which possess tannase activity (12–14). The proposed biochemical pathway for the degradation of tannins by *L. plantarum* implies the action of a tannase and a gallate decarboxylase to decarboxylate the gallic acid formed by tannase action (9, 10). Of the genes involved in tannin degradation in *L. plantarum*, only the gene encoding tan-

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description/relevant marker(s)[a]	Source or reference[b]
Strains		
Escherichia coli		
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacIZYA-argF*) *recA1 gyrA endA1 relA1 supE44 hsdR17*	Clontech
BL21(DE3)	*E. coli* B F$^-$ *dcm ompT hsdS*$_B$(r$_B^-$ m$_B^-$), *gal* λ (DE3)	Novagen
JW2308-4 (CGSC 9853)	Δ(*araD-araB*)*567* Δ*lacZ4787* (::*rrnB-3*) *rph-1* λ$^-$ Δ(*rhaD-rhaB*)*568* Δ*ubiX732*(*del*)::*kan*	31
Lactobacillus plantarum		
ATCC 14917^T	Wild-type strain	CECT
WCFS1	Wild-type strain	M. Kleerebezem
WCFS1ΔlpdB	WCFS1 derivative, Δ*lpdB*	This study
WCFS1ΔlpdC	WCFS1 derivative, Δ*lpdC*	This study
WCFS1ΔlpdD	WCFS1 derivative, Δ*lpdD*	This study
Other		
Enterococcus faecium CECT 410^T	Wild-type strain	CECT
Enterococcus faecium CECT 4102	Wild-type strain	CECT
Lactobacillus brevis CECT 4121^T	Wild-type strain	CECT
Lactobacillus brevis CECT 5354	Wild-type strain	CECT
Lactobacillus hilgardii RM62	Wild-type strain	42
Lactobacillus hilgardii RM63	Wild-type strain	42
Lactobacillus pentosus DSM 20314^T	Wild-type strain	DSMZ
Lactobacillus sakei DSM 15831^T	Wild-type strain	DSMZ
Leuconostoc mesenteroides CECT 219^T	Wild-type strain	CECT
Oenococcus oeni CECT 4100^T	Wild-type strain	CECT
Oenococcus oeni RM1	Wild-type strain	42
Pediococcus pentosaceus CECT 4695^T	Wild-type strain	CECT
Streptococcus gallolyticus UCN34	Wild-type strain	P. Glaser
Plasmids		
pIN-III-A3	Expression vector for producing proteins in *E. coli*	30
pURI3	Expression vector for producing His-tagged proteins in *E. coli*; pT7-7 derivative, Amp^r	42
pIN-lpdB	pIN-III-A3 carrying *lpdB*	This study
pIN-lpdC	pIN-III-A3 carrying *lpdC*	This study
pIN-lpdD	pIN-III-A3 carrying *lpdD*	This study
pURI3-lpdB	pURI3-carrying *lpdB*	This study
pURI3-lpdC	pURI3-carrying *lpdC*	This study
pUCE191	*L. plantarum* integrative vector, pUC19 derivative, Amp^r Em^r Lm^r	18
pUCE191-lpdB	pUCE191 carrying an internal fragment of *lpdB*	This study
pUCE191-lpdC	pUCE191 carrying an internal fragment of *lpdC*	This study
pUCE191-lpdD	pUCE191 carrying an internal fragment of *lpdD*	This study

[a] Amp^r, ampicillin resistance; Em^r, erythromycin resistance; Cm^r, chloramphenicol resistance.
[b] CECT, Spanish Type Culture Collection; DSMZ, German Collection of Microorganisms and Cell Cultures.

nase has been identified so far (15), and the gene encoding the gallate decarboxylase enzyme involved in this degradation remains unknown. In this work, the genes involved in *L. plantarum* gallate decarboxylation have been identified. For the first time, a gallate decarboxylase enzyme has been molecularly identified and characterized. In addition, our results provide new important insights into bacterial nonoxidative aromatic acid decarboxylases.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. plantarum* WCFS1, used throughout this study, was kindly provided by Michiel Kleerebezem (NIZO Food Research, The Netherlands). This strain is a colony isolate of *L. plantarum* NCIMB 8826, which was isolated from human saliva. *L. plantarum* WCFS1 derivative strains, lactic acid bacteria, and the *Escherichia coli* strains used in this study are described in Table 1. *Escherichia coli* JW2308-4 (CGSC 9853), which has a deletion in the *ubiX* gene, was generously provided by the *E. coli* Genetic Stock Center.

Lactic acid bacteria were routinely grown on MRS broth. When gallate activity was assayed and in order to avoid the presence of phenolic compounds included in nondefined medium, bacteria were cultivated in a modified basal medium, RPM, described previously (16). The sterilized modified basal medium was supplemented with filter-sterilized gallic or protocatechuic acid at a 3 mM final concentration. Where appropriate, erythromycin was added to the culture medium at 10 μg/ml. The inoculated media were incubated at 30°C in the dark, without shaking, for 7 to 10 days. Incubated media with cells and without phenolic compound were used as controls. The phenolic compounds present in the supernatants were extracted by a standard protocol involving two extraction steps with one-third of the reaction mixture volume of ethyl acetate.

E. coli cells were routinely grown in LB medium (17) at 37°C with agitation. The *E. coli* JW2308-4 strain was grown in medium containing kanamycin at 30 μg/ml. *E. coli* transformant cells harboring recombinant plasmids were selected on LB medium plates supplemented with 100 μg of ampicillin or 200 μg of erythromycin per ml.

Molecular biology techniques. Standard molecular biology techniques were performed as previously described (17). Plasmid DNA was extracted by a High Pure plasmid isolation kit (Roche). PCR products were purified with a QIAquick gel extraction kit (Qiagen). All cloned

inserts and DNA fragments were confirmed by DNA sequencing with fluorescently labeled dideoxynucleotide terminators and AmpliTaq FS DNA polymerase (Applied Biosystems) in an ABI Prism 377 automated DNA sequencer (Applied Biosystems). Transformation of *E. coli* cells was carried out by using the RbCl method (17). Oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany) (see Table S1 in the supplemental material). Proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Coomassie stained according to standard protocols (17). The protein concentration was determined by using a Bio-Rad protein assay.

***L. plantarum* cell extract preparations.** To identify the protein involved in gallate decarboxylation, cell extracts containing all soluble proteins were prepared. *L. plantarum* WCFS1 was grown in MRS medium at 30°C until exponential phase (optical density at 600 nm [OD_{600}], 0.6). The culture was induced by adding 3 mM gallic acid and incubated for 1 h at 30°C. Uninduced culture was prepared as a control. In the experiments with *L. plantarum lpd* knockout mutants, the mutants were grown in MRS medium containing 10 μg/ml erythromycin until the OD_{600} was 0.6. In all experiments, after incubation the cells were harvested by centrifugation, washed three times with phosphate buffer (50 mM, pH 6.5), and subsequently resuspended in the same buffer for cell rupture. Bacterial cells were disintegrated three times by using a French press at a 1,100-lb/in^2 pressure (Amicon French pressure cell; SLM Instruments). The cell disruption steps were carried out on ice to ensure the low-temperature conditions required for most enzymes. The disintegrated cell suspension was centrifuged at 12,000 × *g* for 20 min at 4°C in order to sediment the cell debris. The supernatant containing the soluble proteins was filtered aseptically using sterile filters with a pore size of 0.2 μm (Sarstedt, Germany).

Gallate decarboxylase assay. The gallate decarboxylase activity of the bacterial cultures was assayed by growing the strains in RPM or LB medium supplemented with 3 mM gallic acid in the dark for several days. The phenolic compounds present in the supernatant were extracted with ethyl acetate as described above. In *E. coli* cultures expressing recombinant *L. plantarum lpd* proteins, gallate decarboxylase activity was assayed in cell extracts incubated at 37°C for 1 h in the presence of 3 mM gallic acid. Similarly, cell extracts were used to assay the gallate decarboxylase activity of *L. plantarum lpd* knockout mutants incubated for 18 h in 3 mM gallic acid. Recombinant His-tagged proteins purified in 50 mM phosphate buffer, pH 6.5, containing 300 mM NaCl, 150 mM imidazole, 1 mM dithiothreitol (DTT), and 50 mM $Na_2S_2O_5$ were assayed for gallate decarboxylase activity (6). Eluted purified proteins (100 μg) were incubated at 37°C in the presence of 3 mM gallic acid and 50 mM L-ascorbate for 1 h.

The reaction products were extracted with ethyl acetate and analyzed by high-pressure liquid chromatography (HPLC) with a diode array detector. A Thermo chromatograph (Thermo Electron Corporation, Waltham, MA) equipped with a P400 SpectraSystem pump, an AS3000 autosampler, and a UV6000LP photodiode array detector was used. A gradient of solvent A (water and acetic acid, 98:2, vol/vol) and solvent B (water, acetonitrile, and acetic acid, 78:20:2, vol/vol/vol) was applied to a reversed-phase Nova-pack C_{18} cartridge (25 cm by 4.0 mm [inner diameter]; particle size, 4.6 μm) at room temperature as follows: 0 to 55 min, 0 to 80% solvent B, linear, 1.1 ml/min; 55 to 57 min, 80 to 90% solvent B, linear, 1.2 ml/min; 57 to 70 min, 90 to 95% solvent B, isocratic, 1.2 ml/min; 70 to 80 min, 95 to 100% solvent B, linear, 1.2 ml/min; 80 to 90 min, 100% linear, 1.2 ml/min; 100 to 120 min, washing with methanol 1.0 ml/min; and reequilibration of the column under initial gradient conditions. Detection was performed by scanning from 220 to 380 nm. Samples were injected onto the cartridge in duplicate, after being filtered through a 0.45-μm-pore-size polyvinylidene difluoride filter. The identification of degradation compounds was carried out by comparing the retention times and spectral data of each peak with those of standards from commercial suppliers.

Construction of *L. plantarum lpd* knockout mutant strains. To ascertain the participation of particular *lpd* genes in gallate decarboxylase activity, insertion-duplication mutagenesis was employed. Internal fragments from the *lpdB* (*lp_0271*), *lpdC* (*lp_2945*), and *lpdD* (*lp_0272*) genes were cloned into the pUCE191 suicide vector. Plasmid pUCE191 is a pUCE19 derivative that does not replicate in *L. plantarum* but expresses lincomycin resistance upon integration into the lactobacillus genome. Plasmid pUCE191 was constructed by introducing the LnR gene from plasmid pFB9 into pUC19 (18). When pUCE191 and its derivatives were used as donor DNA, *L. plantarum* transformants were selected by plating with 10 μg/ml erythromycin and *E. coli* transformants were selected by plating with ampicillin at 100 μg/ml. Plasmids pUCE191-lpdB, pUCE191-lpdC, and pUCE191-lpdD (Table 1), constructed in *E. coli*, were used to transform *L. plantarum* WCFS1 competent cells by electroporation (19). Knockout mutants were selected by plating in MRS medium plates containing erythromycin. The correct insertion of the donor pUCE191 derivative plasmid into the *L. plantarum* WCFS1 chromosome was checked by PCR analysis using primers flanking the target region combined with vector-specific primers (primers 1131 and 1233 for *lpdB*, 388 and 1224 for *lpdC*, and 1109 and 1224 for *lpdD*) (see Table S1 in the supplemental material).

Expression of the *lpd* genes in *E. coli* and purification of the His_6-tagged *lpdB* and *lpdC* recombinant enzymes. The *lpdB*, *lpdC*, and *lpdD* genes from *L. plantarum* WCFS1 were PCR amplified by using HS Prime Start DNA polymerase (TaKaRa) and primer pairs 455 and 390 (*lpdB*), 454 and 388 (*lpdC*), and 1141 and 1142 (*lpdD*). The purified PCR products were inserted into the pIN-III-A3 vector following the restriction enzyme- and ligation-free cloning strategy described previously (20, 21). The procedure used to clone *lpdB* and *lpdC* containing an amino-terminal His_6 tag into the pURI3 vector was essentially the same as that described above for the native protein, but primers 389 and 390 and primers 387 and 388, respectively, were used. The pURI3 vector is a pT7-7 derivative that was used to overproduce recombinant proteins containing a six-histidine tag at their N termini (20).

Cells carrying the recombinant plasmids were grown at 37°C in LB medium containing ampicillin (100 μg/ml), until they reached an optical density at 600 nm of 0.4, and induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside; final concentration, 0.4 mM). After induction, the cells were grown at 22°C for 20 h and collected by centrifugation. Cells were resuspended in phosphate buffer (50 mM, pH 6.5). Crude extracts were prepared by French press lysis of the cell suspension (three times at 1,100 lb/in^2). The insoluble fraction of the lysate was removed by centrifugation at 47,000 × *g* for 30 min at 4°C, and the supernatant was filtered through a 0.2-μm-pore-size filter.

For purification of the recombinant His-tagged LpdB and LpdC proteins, the cultures were similarly prepared but the cells were resuspended in 50 mM phosphate buffer, pH 6.0, containing 30 mg/ml $FeSO_4$, 1 mM DTT, 1 mM L-ascorbate, and 50 mM $Na_2S_2O_5$ (6). The supernatants were applied to a Talon Superflow resin (Clontech) equilibrated with the buffer described above containing 0.3 M NaCl and 10 mM imidazole to improve the interaction specificity in the affinity chromatography step. The bound enzymes were eluted using 150 mM imidazole in the same buffer. The purity of the enzymes was determined by SDS-PAGE in Tris-glycine buffer. Fractions containing the His_6-tagged protein were pooled and analyzed for gallate decarboxylase activity. Eluted purified LpdB and LpdC proteins (100 μg) were incubated at 37°C in the presence of 3 mM gallic acid for 1 h.

***In vitro* protein-protein cross-linking experiments.** The LpdB-LpdC interaction was assayed by glutaraldehyde cross-linking. For glutaraldehyde treatment, LpdB and LpdC proteins, at concentrations ranging from 2 and 10 μM, in 50 mM sodium phosphate, 300 mM NaCl, and 150 mM imidazole buffer (pH 7) were treated with glutaraldehyde solution (0.1, 0.2, and 0.5 μM) for 20 min at room temperature. As a control, a similar glutaraldehyde treatment was applied to monomeric lysozyme. The reactions were stopped by adding Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, β-mercaptoethanol, 10% glycerol) containing 0.005% bromophenol blue. Samples were separated by SDS-PAGE and revealed by Coomassie blue staining.

PCR detection of gallate decarboxylase. Genes encoding LpdB and LpdC were amplified by PCR using chromosomal DNA from several lactic acid bacterial strains. Amplifications were performed by using degenerate primers 450 and 451 and degenerate primers 448 and 449 to amplify *lpdB* and *lpdC*, respectively. These degenerate primers were based on the well-conserved domains of the B and C proteins. The reactions were performed in a Personal thermocycler (Eppendorf), using 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 30 s. The expected sizes of the amplicons were 158 bp (subunit B) and 300 bp (subunit C). Amplified fragments were resolved on 2% agarose gels.

Protein identification via MS. The protein band was excised manually and then digested automatically using a Proteineer DP protein digestion station (Bruker-Daltonics, Bremen, Germany). The protocol described by Shevchenko et al. (22) was used for trypsin digestion. The digestion was analyzed in an Ultraflex time-of-flight mass spectrometer (MS; Bruker-Daltonics) equipped with a LIFT-MS/MS device. Spectra were acquired in the positive-ion mode at a 50-Hz laser frequency, and 100 to 1000 individual spectra were averaged. Automated analysis of mass data was performed using flexAnalysis software (Bruker-Daltonics). Matrix-assisted laser desorption ionization MS and MS/MS data were combined through the BioTools program (Bruker-Daltonics) to search a nonredundant protein database (NCBInr Swiss-Prot) using Mascot software (Matrix Science, London, United Kingdom).

Sequence data analyses. A homology search with finished and unfinished microbial genome databases was performed with the TBLAST algorithm at the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). Multiple alignments were made using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/) on the EBI site, after retrieval of sequences from the GenBank and Swiss-Prot databases.

RESULTS

Identification of the enzyme responsible for gallate decarboxylation in *L. plantarum* WCFS1. In order to know if gallate decarboxylase is an inducible enzyme, mid-exponential-phase *L. plantarum* WCFS1 cultures were incubated in MRS medium containing glucose as the carbon source, with or without the addition of 3 mM gallic acid for 1 h at 30°C. Cell extracts prepared from these cultures were tested for activity on gallic acid. The extract from the control culture (grown in the absence of gallic acid) did not show decarboxylase activity. However, the extract from the culture grown in the presence of gallic acid for 1 h was able to decarboxylate the gallic acid present in the reaction mixture (data not shown). Similar to the result for *p*-coumaric acid decarboxylase previously described in *L. plantarum* (23, 24), this result might indicate that gallate decarboxylase is an inducible enzyme.

Cell extracts were resolved by SDS-PAGE in order to find proteins overproduced from the induced culture (Fig. 1). The only difference clearly detected was in the gallate-induced culture and consisted of a protein band of approximately 50 kDa which was absent in the uninduced sample. The overproduced protein was excised from the gel, and its identification was done by in-gel trypsin and chymotrypsin digestion and subsequent mass spectrometry analyses. The result obtained unambiguously identified the protein as 3-octaprenyl-4-hydroxybenzoate carboxy-lyase or UbiD (NP_786283), encoded by the *lp_2945* locus.

In *E. coli*, the UbiD protein is involved in the biosynthesis of ubiquinone (or coenzyme Q) (25). Ubiquinone plays an essential role in aerobic respiratory electron transfer for energy generation. The biosynthesis of ubiquinone involved at least nine reactions. In one of these reactions, the 3-octaprenyl-4-hydroxybenzoate is decarboxylated to 2-octaprenylphenol by the enzyme 3-octaprenyl-4-hydroxybenzoate decarboxylase. There are two isofunctional enzymes in *E. coli* K-12, UbiD and UbiX, which catalyze this reaction (26). Their amino acid sequences share no similarity. UbiX, a 21-kDa protein, may require a flavin nucleotide as a cofactor, whereas UbiD is a 55-kDa protein requiring divalent metal for activity. Of the two enzymes, UbiD accounts for almost 80% of the total activity (27). However, it has been described that *L. plantarum* does not produce mena- or ubiquinones, as it needs the exogenous addition of at least menaquinones for heme-assisted respiration (28). Therefore, it is unlikely that UbiD is involved in the self-production of ubiquinones by *L. plantarum*. The precise biochemical function of the *L. plantarum* protein annotated UbiD (Lp_2945) is likely to be a gallate decarboxylase.

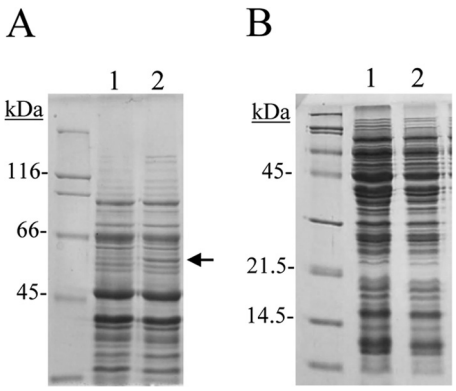

FIG 1 SDS-PAGE analysis of protein extracts from *L. plantarum* WCFS1 grown in the presence of 3 mM gallic acid. Lanes 1, uninduced cell extracts; lanes 2, extracts induced with gallic acid for 1 h. The arrow indicates the induced protein. The 8% (A) and 15% (B) gels were stained with Coomassie blue. Molecular mass markers are located on the left (SDS-PAGE standards; Bio-Rad).

Genes encoding *L. plantarum* gallate decarboxylase. Recent studies indicated that *ubiD*-like genes in many prokaryotes are located within operons that encode partner proteins, including proteins homologous to UbiX, which are required to decarboxylate a variety of hydroxyarylic or aromatic acids. Bacterial nonoxidative, reversible multisubunit hydroxyarylic acid decarboxylases/phenol carboxylases are encoded by three clustered genes (genes encoding the B, C, and D subunits) (29). The corresponding genes from *Sedimentibacter hydroxybenzoicus*, *Bacillus subtilis*, *Streptomyces* sp. strain D7, *E. coli* O157:H7, *K. pneumoniae*, and *Salmonella enterica* serovar Typhimurium were cloned and expressed in *E. coli* and shown to code for proteins exhibiting nonoxidative aromatic acid decarboxylase activity (29). Database searches revealed the existence of different gene organizations among these decarboxylases: the BCD gene arrangement (such as in *Enterobacter cloacae*, *Pantoea ananatis*, *B. subtilis*, and a *Streptomyces* sp.), CDB (*S. hydroxybenzoicus*, *Clostridium* sp. strain D5, and *Olsenella uli* DSM 7084), CDB in several lactic acid bacteria (*Oenococcus oeni*, *Lactobacillus brevis*, and *Enterococcus faecium*), and DBC in *S. gallolyticus* (Fig. 2). Surprisingly, the genes that putatively encoded gallate decarboxylase, the *lpd* (from *Lactobacillus plantarum* decarboxylase, to be consistent with the existing nomenclature of genes encoding aromatic acid decarboxylases) genes, are not close only in the genome of *L. plantarum*. The gene encoding the C subunit, *lpdC* or *lp_2945*, which was overproduced in the presence of gallate, is located close to the gene encoding tannase (*tanLp1* or *lp_2956*). However, the genes encoding the B (*lp_0271*) and D

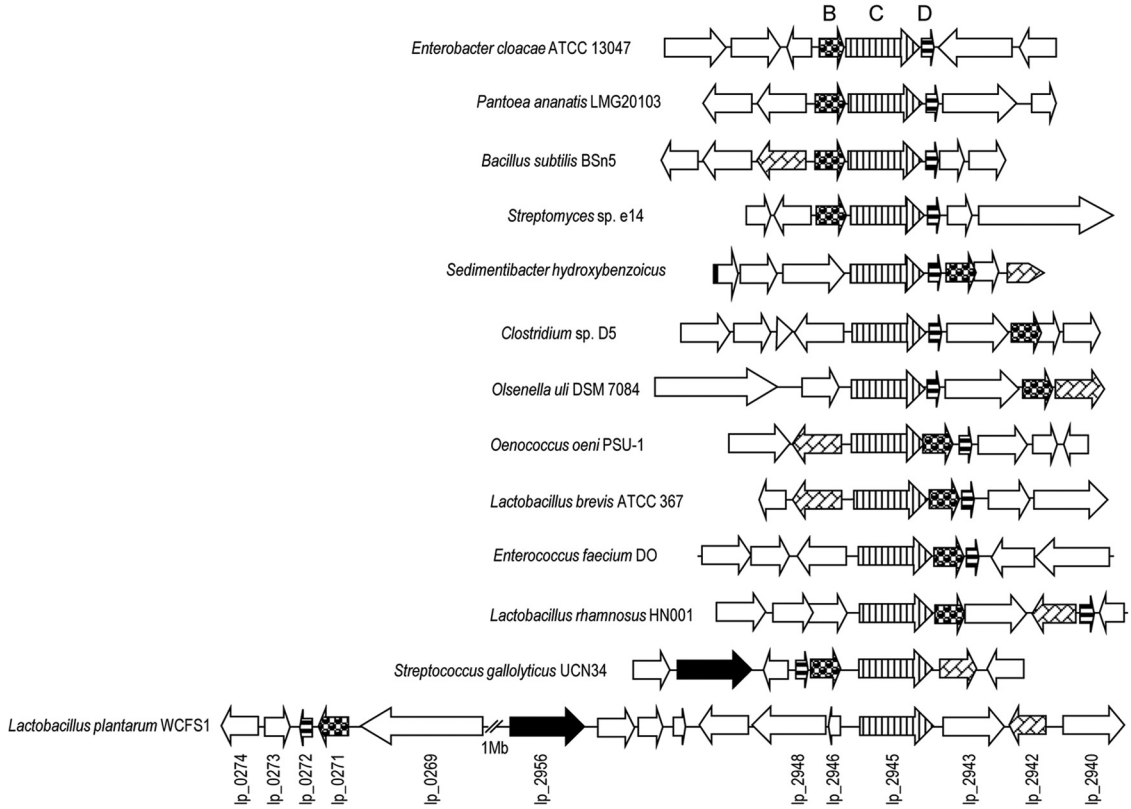

FIG 2 Genetic organization of the *L. plantarum* WCFS1 chromosomal region containing the genes encoding gallate decarboxylase (GenBank accession no. NC_004567, positions 243093 to 252815 and 2618290 to 2635122). The genetic organization from several bacterial nonoxidative aromatic acid decarboxylases (B, C, and D subunits) is also represented, such as those of *Enterobacter cloacae* subsp. *cloacae* ATCC 13047 (GenBank accession no. NC_014121, positions c/4181733 to 4189439 [where "c/" indicates positions on a complementary strand]), *Pantoea ananatis* LMG20103 (GenBank accession no. NC_013956, positions 1814209 to 1820127), *Bacillus subtilis* BSn5 (GenBank accession no. NC_014976, positions 2624421 to 2630650), *Streptomyces* sp. strain e14 (GenBank accession no. NZ_ACUR00000000, positions c/7010912 to 7018020), *Sedimentibacter hydroxybenzoicus* (GenBank accession no. AF128880, positions 1 to 5796), *Clostridium* sp. D5 (GenBank accession no. NZ_ADBG00000000, positions 5032836 to 5040967), *Olsenella uli* DSM 7084 (GenBank accession no. NC_014363, positions c/1673164 to 1680578), *Oenococcus oeni* PSU-1 (GenBank accession no. NC_008528, positions c/1104544 to 1114618), *Lactobacillus brevis* ATCC 367 (GenBank accession no. NC_008497, positions c/1942529 to 1951428), *Enterococcus faecium* DO (GenBank accession no. NC_017960, positions 1147858 to 1155656), *Lactobacillus rhamnosus* HN001 (GenBank accession no. NZ_ABWJ00000000, positions 1905984 to 1914300), and *Streptococcus gallolyticus* UCN34 (GenBank accession no. NC_013798, positions 1699210 to 1708634). Arrows indicate genes. Genes having putative identical functions are represented by identical shading. The genes having brick-like shading encode putative LysR-type transcriptional regulators. Genes coding for putative tannase proteins are represented by black arrows.

(*lp_0272*) subunits are located more than 1 Mb apart in the *L. plantarum* genome. This unusual gene organization could indicate a different catalytic mechanism of *L. plantarum* gallate decarboxylase.

Pyrogallol production by recombinant *E. coli* cells harboring *lpdB*, *lpdC*, and *lpdD* genes. In order to know the catalytic subunits involved in gallate decarboxylation, the *lpd* genes were individually cloned into the expression vector pIN-III-A3 under the control of the lpp^{P}-5 and lac^{PO} promoters, which can be induced to high levels with IPTG (30). The correct sequence of the recombinant plasmids pIN-lpdB, pIN-lpdC, and pIN-lpdD was verified by DNA sequencing.

Cell extracts were prepared from *E. coli* DH5α cells harboring the recombinant plasmids. The extracts were used to detect the presence of hyperproduced proteins. Control cells containing the pIN-III-A3 vector plasmid alone did not show expression over the time course analyzed (overnight), whereas expression of additional 54-, 21-, and 15-kDa proteins was apparent in DH5α cells harboring pIN-lpdC, pIN-lpdB, and pIN-lpdD, respectively (Fig. 3A and B). These molecular masses are in good agreement with the relative molecular masses deduced from the nucleotide sequences of the *lpdC*, *lpdB*, and *lpdD* genes.

Extracts from *E. coli* cells carrying the pIN-III-A3, pIN-lpdC, pIN-lpdB, and pIN-lpdD plasmids adjusted to the same protein concentration were assayed for gallate decarboxylase activity. Reactions with mixtures containing the same total protein concentration were done by mixing these extracts in all possible combinations, e.g., in mixtures containing the B, C, or D subunit individually; mixtures containing subunits B and C, B and D, and C and D; and finally, mixtures simultaneously containing the three different *lpd* subunits. Reactions were carried out for 1 h, and after that, the phenolic compounds present in the reaction mixtures were extracted by ethyl acetate and analyzed by HPLC.

Figure 3C shows that all the reactions with mixtures containing LpdC were able to partially decarboxylate gallic acid to a similar extent. In contrast, in reactions in which LpdC was absent from the reaction mixture, gallic acid was not metabolized. These results indicate the involvement of LpdC in the catalysis of decar-

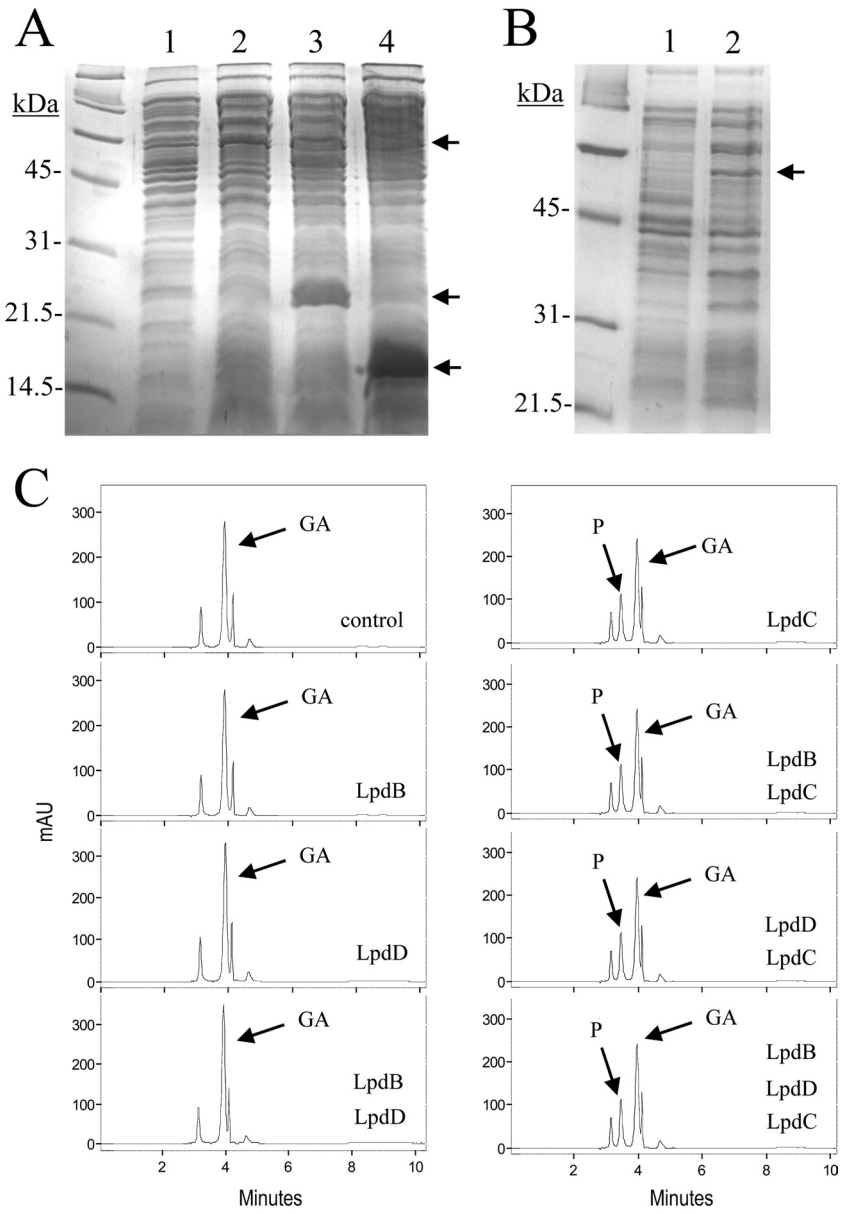

FIG 3 Pyrogallol production by cell extracts from recombinant *E. coli* cells harboring *lpdB*, *lpdC*, and *lpdD* genes. (A and B) SDS-PAGE analysis of cell extracts of IPTG-induced cultures of *E. coli* DH5α bearing recombinant pIN-III-A3 plasmids for LpdB, LpdC, and LpdD protein production. (A) LpdC, LpdB, and LpdD production (15% gel). Lane 1, *E. coli* DH5α(pIN-III-A3); lane 2, *E. coli* DH5α(pIN-lpdC); lane 3, *E. coli* DH5α(pIN-lpdB); lane 4, *E. coli* DH5α(pIN-lpdD). (B) LpdC production (12% gel). Lane 1, *E. coli* DH5α(pIN-III-A3); lane 2, *E. coli* DH5α(pIN-lpdC). The arrows indicate the overproduced proteins. The gels were stained with Coomassie blue. Molecular mass markers are located on the left. (C) Gallate decarboxylase activity of *E. coli* DH5α cell extracts harboring *lpdB*, *lpdC*, and *lpdD* genes. HPLC chromatograms of *E. coli* cell extracts, at the same total protein concentration, incubated in 3 mM gallic acid for 1 h: pIN-III-A3 (control), pIN-III-A3 plus pIN-lpdB (LpdB), pIN-III-A3 plus pIN-lpdD (LpdD), pIN-III-A3 plus pIN-lpdB and pIN-lpdD (LpdB LpdD), pIN-III-A3 plus pIN-lpdC (LpdC), pIN-III-A3 plus pIN-lpdB and pIN-lpdC (LpdB LpdC), pIN-III-A3 plus pIN-lpdC and pIN-lpdD (LpdC LpdD), and pIN-lpdB plus pIN-lpdC and pIN-lpdD (LpdB LpdC LpdD). The gallic acid (GA) and pyrogallol (P) detected are indicated. Chromatograms were recorded at 280 nm. mAU, milli-absorbance units.

boxylation, and in addition, they suggest that LpdC is the only subunit required to yield gallate decarboxylase activity.

From the results obtained using *E. coli* extracts, the possibility that in *E. coli* the missing subunits can be replaced by other *E. coli* proteins, e.g., UbiX, to yield enzyme activity could not be excluded. As explained before, UbiX is involved in ubiquinone biosynthesis and catalyzes the reaction of 3-octaprenyl-4-hydroxybenzoate to 2-octaprenylphenol. In order to avoid the presence of a functional *E. coli* UbiX protein in the extracts, plasmid pIN-lpdC was introduced into *E. coli* JW2308-4 (CGSC 9853), a UbiX-defective mutant (31). Gallate decarboxylase activity was assayed in cell extracts prepared from *E. coli* JW2308-4 harboring pIN-lpdC. The results indicated that, similar to *E. coli* DH5α extracts, pyrogallol was produced from gallic acid in the presence of LpdC even in the absence of a functional *E. coli* UbiX protein (see Fig. S1 in the supplemental material). Therefore, the possibility of the in-

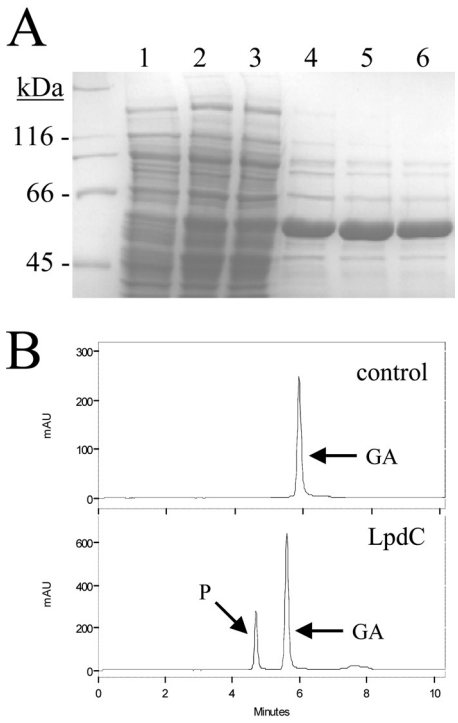

FIG 4 Purification and enzymatic activity of recombinant *L. plantarum* LpdC protein. (A) SDS-PAGE analysis of the expression and purification of the His_6-tagged LpdC. Results of analysis of soluble cell extracts of IPTG-induced *E. coli* BL21(DE3)(pURI3) (lane 1) or *E. coli* BL21(DE3)(pURI3-lpdC) (lane 2), flowthrough from the affinity resin (lane 3), or fractions eluted after His affinity resin (lanes 4 to 6) are shown. The 8% gel was stained with Coomassie blue. Molecular mass markers are located on the left (SDS-PAGE standards; Bio-Rad). (B) Gallate decarboxylase activity of purified His_6-LpdC protein. An HPLC chromatogram of LpdC (100 μg) incubated in 3 mM gallic acid for 1 h (LpdC) is shown. A chromatogram without protein (control) is also shown. The gallic acid (GA) and pyrogallol (P) detected are indicated. Chromatograms were recorded at 280 nm.

volvement of *E. coli* UbiX in the gallate decarboxylase activity observed in *E. coli* extracts could be discarded.

Enzymatic activity of purified His_6-LpdC. To further investigate the decarboxylase activity of LpdC and the possible involvement of *lpdB* or *lpdB*-like genes, His-tagged proteins LpdB and LpdC were constructed for expression and purification from *E. coli*. His_6-tagged proteins were cloned into *E. coli* BL21(DE3), overproduced, and purified by a one-step affinity procedure as described in Materials and Methods. Only the His_6-LpdC protein showed gallate decarboxylase activity. However, even though His_6-LpdC was produced at a high yield, it presented low gallate decarboxylase activity, as only degradation similar to that of the cell extracts was observed (Fig. 4).

In order to know the involvement of LpdB on activity, reactions were performed by using both purified His_6-LpdB and His_6-LpdC proteins. Decarboxylase activity did not increase due to the presence of the LpdB subunit (see Fig. S2 in the supplemental material). Moreover, *in vitro* protein-protein cross-linking experiments using glutaraldehyde did not show physical interaction between the two proteins (see Fig. S3 in the supplemental material).

Effects of disruption of *lpdB*, *lpdC*, and *lpdD* on gallate decarboxylation by *L. plantarum*. To corroborate previous results, insertion-duplication mutagenesis was employed to construct *L. plantarum* mutants with knockouts in the *lpdB*, *lpdC*, and *lpdD* genes. The correct insertion of the donor plasmids into the *L. plantarum* WCFS1 chromosome was verified by PCR. Unexpectedly, when these mutants were grown in the presence of gallic acid, the *lpdB* and *lpdC* mutants were unable to decarboxylate it to pyrogallol (Fig. 5), suggesting the participation of both proteins, LpdB and LpdC, in the decarboxylation of this hydroxybenzoic acid. Taking into account the probable operonic organization of the *lpdBD* genes (Fig. 2), the *lpdB* mutant could, in fact, be a double-knockout mutant. The *lpdD* mutant was the only mutant able to decarboxylate gallic acid. Similar to gallic acid, protocatechuic acid was decarboxylated in the wild type and disrupted *L. plantarum* cells, except cells in which the *lpdB* and *lpdC* genes were interrupted (Fig. 5). The results obtained from the *L. plantarum* knockout mutants indicate that the B and C subunits of the decarboxylase seem to be essential for gallate and protocatechuate decarboxylase activity in *L. plantarum* WCFS1, whereas the D subunit is not involved.

To ascertain the participation of *lpd* genes in gallate decarboxylase activity, cell extracts were prepared from *L. plantarum* wild type and knockout mutants. The extracts were adjusted to the same protein concentration, and, similar to the reactions with the *E. coli* extracts described above, the reactions were done with mixtures in which these extracts were mixed in all possible combinations in the presence of gallic acid. Reactions were carried out for 18 h, and after that, the phenolic compounds present in the reaction mixtures were extracted with ethyl acetate and analyzed by HPLC.

Figure 6 shows that only the reaction with a mixture which contained a functional copy of the *lpdB* and *lpdC* genes from the same strain was able to decarboxylate gallic acid. Surprisingly, gallate decarboxylase activity was not observed when functional LpdB and LpdC proteins came from different extracts. The only explanation for this result could be that LpdB has a possible role during the maturation (e.g., folding) or activation of LpdC, the main catalytic subunit.

Gallate decarboxylase activity in lactic acid bacteria. Once the direct involvement of *lpd* genes in gallate/protocatechuate decarboxylation was demonstrated, as shown in Fig. 2, it seems probable that other species of lactic acid bacteria could also decarboxylate these aromatic acids. The sequences of LpdB and LpdC proteins from nine lactic acid bacteria were aligned. The degree of identity among these LpdB proteins ranged from 56 to 80% (see Fig. S4 in the supplemental material). The identity shown among the LpdC proteins was higher, ranging from 73 to 90% (see Fig. S5 in the supplemental material). In both cases, proteins from *L. plantarum* and *Lactobacillus pentosus* presented a 98% identity. These alignments allowed us to identify conserved amino acid domains to design degenerate oligonucleotides to detect the presence of both genes by PCR. Oligonucleotides 450 and 451 amplify a 158-bp internal fragment of the subunit B gene in lactic acid bacteria; similarly, oligonucleotides 448 and 449 amplify a 300-bp fragment of the gene encoding subunit C.

In order to associate the presence of the *lpdB* and *lpdC* genes and the ability to degrade gallic and protocatechuic acids, selected strains of lactic acid bacteria (Table 1) were grown in culture medium containing these hydroxybenzoic acids, and their supernatants were analyzed for the production of pyrogallol or catechol. In addition, their DNAs were used as the templates in PCRs using oligonucleotides 450 and 451 and oligonucleotides 448 and 449 to detect the presence of the genes encoding subunits B and C, respectively.

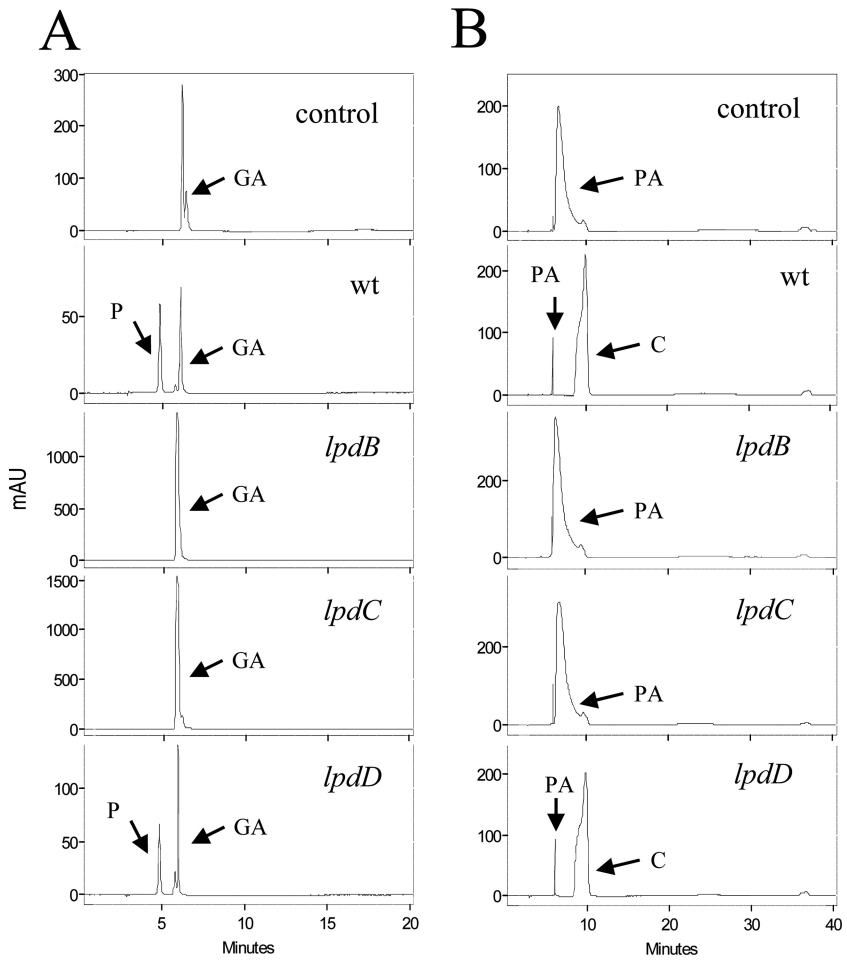

FIG 5 Effects of disruption of *lpdB*, *lpdC*, and *lpdD* on gallate and protocatechuate decarboxylation in *L. plantarum* WCFS1. HPLC chromatograms of *L. plantarum* cultures incubated in 3 mM gallic acid (A) or protocatechuic acid (B) are shown for *L. plantarum* WCFS1 (wild type [wt]), *L. plantarum* WCFS1(pUCE191-lpdB) (*lpdB* mutant), *L. plantarum* WCFS1(pUCE191-lpdC) (*lpdC* mutant), and *L. plantarum* WCFS1(pUCE191-lpdD) (*lpdD* mutant). Results for uninoculated medium are also shown (control). The gallic acid (GA), protocatechuic acid (PA), pyrogallol (P), and catechol (C) detected are indicated. Chromatograms were recorded at 280 nm.

Strains belonging to the species *E. faecium*, *L. brevis*, *L. pentosus*, *L. plantarum*, *O. oeni*, and *S. gallolyticus* amplified fragments from both genes (Fig. 7A and B); *Lactobacillus sakei* DSM 15831^T amplified only the gene encoding subunit C; and finally, strains of the species *Lactobacillus hilgardii*, *Leuconostoc mesenteroides*, and *Pediococcus pentosaceus* did not amplify either of the two genes. These results are mostly in agreement with the information obtained from the complete genome sequences of representative strains of these lactic acid bacterial species. However, unexpected results were obtained with *L. sakei* and *P. pentosaceus*. The genome sequence of *L. sakei* subsp. *sakei* 23K revealed the presence of a copy of the gene encoding subunit B which was absent from the *L. sakei* subsp. *carnosus* DSM 15831^T strain used in this study. Similarly, the genome sequence of *P. pentosaceus* ATCC 25745 revealed the presence of both genes; however, these genes were absent from *Pediococcus claussenii* ATCC BAA-344, as revealed from its sequenced genome, and from *P. pentosaceus* CECT 4695, used in this study. These results might indicate that, at least in these species, the ability to decarboxylate gallic and protocatechuic acids is strain specific.

HPLC analysis of the supernatants from cultures of these bacteria in the presence of gallic or protocatechuic acid indicated that only the bacteria which possess the genes encoding subunits B and C are able to decarboxylate gallic acid to pyrogallol and protocatechuic acid to catechol (Fig. 7C). Strains from the species *E. faecium*, *L. brevis*, *L. pentosus*, *L. plantarum*, and *O. oeni* were able to decarboxylate gallic and protocatechuic acids; however, *L. hilgardii*, *L. mesenteroides*, *L. sakei*, and *P. pentosaceus* strains were not. Therefore, the results obtained seem to indicate that the ability to decarboxylate some hydroxybenzoic acids (gallic and protocatechuic acids) is widely extended among lactic acid bacterial strains. Moreover, the ability to decarboxylate these acids is related to the presence of the B and C subunits of a putative aromatic acid decarboxylase found in these bacteria.

DISCUSSION

Bacterial nonoxidative, reversible multisubunit aromatic acid decarboxylases are encoded by three clustered genes, B, C, and D. The functions of these proteins remain unknown, and the question that arises is, which genes encode the catalytic protein? Initially, when

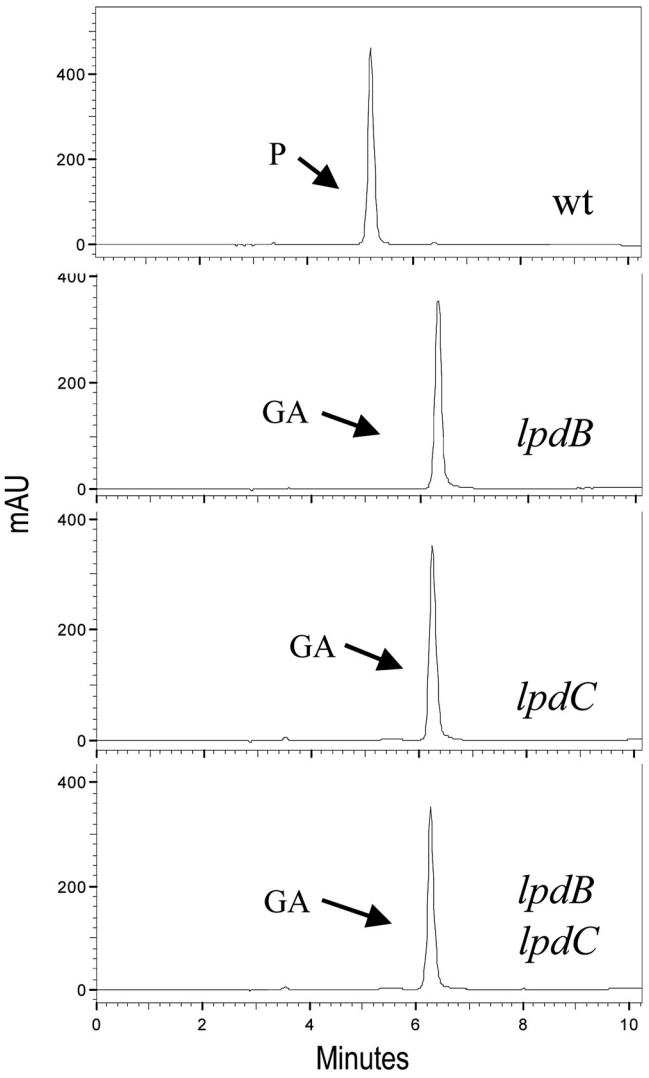

FIG 6 Pyrogallol production by cell extracts from *L. plantarum* knockout mutants. HPLC chromatograms of *L. plantarum* cell extracts, at the same total protein concentration, incubated in 3 mM gallic acid for 18 h are shown for *L. plantarum* WCFS1 (wild type), *L. plantarum* WCFS1(pUCE191-lpdB) (*lpdB* mutant), *L. plantarum* WCFS1(pUCE191-lpdC) (*lpdC* mutant), and *L. plantarum* WCFS1(pUCE191-lpdB) plus *L. plantarum* WCFS1 (pUCE191-lpdC) (*lpdB* and *lpdC* mutants). The gallic acid (GA) and pyrogallol (P) detected are indicated. Chromatograms were recorded at 280 nm.

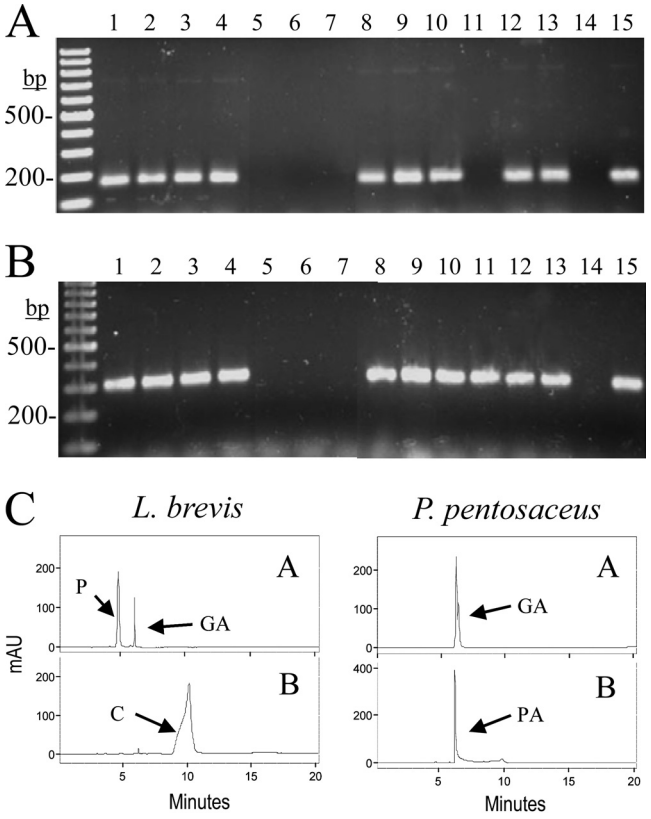

FIG 7 Gallate and protocatechuate decarboxylase activity in lactic acid bacteria. (A and B) PCR amplification of the B and C subunits of putative gallate decarboxylases. Chromosomal DNA from the following strains was used for PCR amplification with oligonucleotides 450 and 451 (A) or oligonucleotides 448 and 449 (B) to amplify 158 bp or 300 bp of the B or C subunit, respectively: *E. faecium* CECT 410^T (lane 1), *E. faecium* CECT 4102 (lane 2), *L. brevis* CECT 4121^T (lane 3), *L. brevis* CECT 5354 (lane 4), *L. hilgardii* RM62 (lane 5), *L. hilgardii* RM63 (lane 6), *L. mesenteroides* CECT 219^T (lane 7), *L. pentosus* DSM 20314^T (lane 8), *L. plantarum* ATCC 14917^T (lane 9), *L. plantarum* WCFS1 (lane 10), *L. sakei* DSM 15831^T (lane 11), *O. oeni* CECT 4100^T (lane 12), *O. oeni* RM1 (lane 13), *P. pentosaceus* CECT 4695^T (lane 14), and *S. gallolyticus* UCN34 (lane 15). PCR products were subjected to gel electrophoresis and stained with Gel Red. Left lane, 100-bp molecular size ladder. Numbers indicate some of the molecular sizes. (C) HPLC chromatograms of supernatants from lactic acid bacteria. The *L. brevis* CECT 5354 and *P. pentosaceus* CECT 4695^T strains were grown in RMP medium containing 3 mM gallic acid (A) or protocatechuic acid (B) for 10 days. The gallic acid (GA), protocatechuic acid (PA), pyrogallol (P), and catechol (C) detected are indicated. Chromatograms were recorded at 280 nm.

these decarboxylases were purified from cell extracts in an active form, the results indicated that only one multimeric protein, composed of identical subunits, was involved. Purified 4-hydroxybenzoate decarboxylase from *S. hydroxybenzoicus* showed a single band on both native gradient PAGE and denatured SDS-PAGE, having an apparent molecular mass of 350 kDa and consisted of six identical subunits of 57 kDa (32). Similarly, in *P. agglomerans*, SDS-PAGE analysis indicated that the purified gallate decarboxylase was homogeneous and consisted of six identical subunits of 57 kDa in molecular mass (6). In addition, purified 4-hydroxybenzoate decarboxylase from *E. cloacae* was a homohexamer of identical 60-kDa subunits (33). The molecular masses of these proteins are in accordance with the masses of their respective C subunits deduced from *in silico* sequence analysis.

However, when the genetic organization of these proteins was known, results involving the activity of the additional B and D subunits were obtained. Contradictory results about the involvement of the B, C, and D subunits in the activity of these decarboxylases were obtained (29, 32, 34, 35). It seems that purification from cell extracts indicated that, on the basis of the size of the purified protein, only the C subunit is involved in enzymatic activity; however, experiments of heterologous expression indicated that the three genes, or at least subunits C and D, are needed for activity (29, 36). It was also speculated that during heterologous expression in *E. coli*, the B subunit can be at least partially replaced by another gene product from *E. coli*, such as UbiX (36).

As shown in Fig. 2, genes encoding nonoxidative decarboxylase are clustered in different organizations in bacteria. However, *L. plantarum* is the only bacterial species in which the genes are sep-

arated in the chromosome by more than 1 Mb. This unusual configuration could indicate a different enzymatic organization. The first result suggesting the involvement of LpdC in gallate decarboxylase activity in *L. plantarum* was that, in induced cell extracts, only this protein was significantly overproduced, as observed in the 8 and 15% SDS-PAGE analysis (Fig. 1). In addition, only the LpdC protein was overproduced (more than 7-fold) in *L. plantarum* when it was exposed to a tannic acid challenge, as revealed by a proteomic analysis (37). Similarly, only the proteins equivalent to LpdC were overproduced in response to other phenolic acids, such as protein 3717 (VdcC) in *Streptomyces* sp. D7 upon exposure to vanillic acid (34) and BsdC in *B. subtilis* in response to salicylic acid (38). However, in *Streptomyces* sp. D7 as well as in *B. subtilis*, it has been described that at least subunits C and D are required to confer decarboxylase activity (29, 36).

The only involvement of LpdC in *L. plantarum* decarboxylation also arose from *E. coli* extracts producing LpdB, LpdC, and LpdD proteins. The three proteins were independently overproduced in *E. coli*, and the expression of *lpdC* was enough to confer gallate decarboxylase activity to *E. coli*, even in an *E. coli* UbiX-defective mutant. Finally, to ascertain the exclusive role of LpdC in gallate decarboxylase activity, the recombinant LpdC protein was purified and gallate decarboxylase activity was demonstrated *in vitro*. However, even though LpdC was produced in a high yield, it presented low gallate decarboxylase activity. It is noteworthy that addition of pure LpdB protein did not increase the activity of LpdC. It could be argued that the presence of a His tag could result in differences in activity. More likely, the low activity observed could be because these enzymes are extremely unstable in usual buffer solutions due to their oxygen sensitivity (6, 7). In addition, the batch purification protocol followed for protein purification and the presence of immobilized metal ions (cobalt) in the resin could contribute to the inactivation of the enzyme.

Unexpected results were obtained by the use of *L. plantarum* mutants with knockouts in the three *lpd* genes, as it was demonstrated that the disruption of subunit B and subunit C avoids gallate decarboxylase activity in *L. plantarum*. Decarboxylase activity was restored only when extracts containing functional B and C proteins were present. These results could be compatible with those obtained from *E. coli* extracts only if a protein from *E. coli* could assume the function of LpdB. In spite of the similarity of UbiX and LpdB, the expression of *lpdC* in a *ubiX*-negative *E. coli* mutant (*E. coli* JW2308-4) indicates that UbiX is not assuming the LpdB function. An unknown *E. coli* protein different from UbiX could be involved. The involvement of the B subunit in the decarboxylation reaction has also been clearly demonstrated in *B. subtilis* since antisense mRNA inactivation of the B subunit highly reduces the enzyme activity to below 2% of that of the wild type (29, 36).

The biochemical activities of the three different protein subunits have not been assigned. So far, it has not been possible to unequivocally correlate genes coding for aromatic acid decarboxylase and their function. In this study, interesting results came from the use of *L. plantarum* knockout mutants. In *L. plantarum*, the LpdD protein did not seem to be necessary for gallate decarboxylase activity, while the LpdC protein seemed to be the main catalytic subunit. However, the function of LpdB is unknown. To achieve gallate decarboxylase activity fully, it is not enough to have functional copies of the LpdB and LpdC proteins, since it seems that both proteins need to be synthesized in the same strain. Both mature proteins do not seem to interact, as revealed by the *in vitro* cross-linking experiments. It is tempting to speculate that LpdB could have a possible role during the maturation (e.g., folding) or activation of LpdC, and therefore, LpdB and LpdC need to be synthesized simultaneously in the same host. The mechanism of decarboxylation followed by these aromatic acid decarboxylases is a paradigm for a new type of biological decarboxylation reaction. As far as we know, this study in *L. plantarum* constitutes the first description of the involvement of only subunits B and C in the nonoxidative decarboxylation of an aromatic acid.

Apart from *E. faecalis*, among lactic acid bacteria, decarboxylation of aromatic acids has been described only in *L. plantarum* (11), *L. brevis* (39), and *S. gallolyticus* (8, 40). These bacteria decarboxylate the same hydroxybenzoic acids, gallic acid and protocatechuic acid, and all possess genes similar to the gallate decarboxylase genes described in this work (Fig. 2). Such decarboxylase activity has never been described in *E. faecium* and *O. oeni* species; however, strains from these species also decarboxylate both acids and possess both genes (Fig. 2 and 7). From the data obtained in this study, it could be assumed that, at least in some bacterial species, the ability to decarboxylate gallic and protocatechuic acids might be strain dependent, similar to the ability of some specific *E. coli* strains (e.g., strains EDL933 and VT2-Sakai from *E. coli* O157:H7) to decarboxylate 4-hydroxybenzoate (29).

The identification of the *L. plantarum* gallate decarboxylase involved in tannin degradation completes the analysis of the first route of degradation of a phenolic compound in lactic acid bacteria. The proposed biochemical pathway for the degradation of tannins by *L. plantarum* implies that tannins are hydrolyzed to gallic acid and glucose by a tannase action, and the gallic acid formed is decarboxylated to pyrogallol by the action of a gallate decarboxylase (9, 10). When purified *L. plantarum* tannase was assayed against 18 phenolic acid esters, only esters derived from gallic and protocatechuic acids were hydrolyzed (41), with these esters apparently sharing the same substrate specificity as the decarboxylase enzyme. This substrate specificity suggests a concomitant activity of tannase and gallate decarboxylase on specific phenolic substrates. This is more obvious when the chromosomal location of these genes is considered. The genes encoding gallate decarboxylase (*lp_2945*) and tannase (*lp_2956*) are only 6.5 kb apart on the *L. plantarum* WCFS1 chromosome. Interestingly, in *S. gallolyticus*, another tannin-degrading lactic acid bacterium, the gene encoding tannase (GALLO_1609) is separated by only one open reading frame from the genes encoding decarboxylase (GALLO_1611, GALLO_1612, and GALLO_1613) (Fig. 2). More interestingly, *S. gallolyticus* strains showed metabolism of these phenolic compounds identical to that of *L. plantarum*, as they hydrolyzed hydrolyzable tannins to release gallic acid, which was subsequently decarboxylated to pyrogallol, and protocatechuic acid, which was decarboxylated to catechol (8, 40). Neither the *S. gallolyticus* nor *L. plantarum* bacterial species possesses appropriate mechanisms to further degrade the compounds produced by these dead-end pathways. The physiological relevance of these reactions is unknown, but in natural ecosystems, it could be imagined that other organisms in a consortium mineralize and remove these dead-end metabolites. These enzymatic activities have ecological advantages for *L. plantarum*, as it is often associated with fermentation of plant materials. Therefore, *L. plantarum* plays an important role when tannins are present in food and the intestine, having the capability of degrading and detoxifying harmful and antinutritional constituents into simpler and innocuous compounds.

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Capítulo 4

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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Characterization of a bacterial tannase from *Streptococcus gallolyticus* UCN34 suitable for tannin biodegradation

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Abstract The gene in the locus GALLO_1609 from *Streptococcus gallolyticus* UCN34 was cloned and expressed as an active protein in *Escherichia coli* BL21 (DE3). The protein was named TanSg1 since it shows similarity to bacterial tannases previously described. The recombinant strain produced His-tagged TanSg1 which was purified by affinity chromatography. Purified TanSg1 protein showed tannase activity, having a specific activity of 577 U/mg which is 41 % higher than the activity of *Lactobacillus plantarum* tannase. Remarkably, TanSg1 displayed optimum catalytic activity at pH 6–8 and 50–70 °C and showed high stability over a broad range of temperatures. It retained 25 % of its relative activity after prolonged incubation at 45 °C. The specific activity of TanSg1 is enhanced by the divalent cation Ca^{2+} and is dramatically reduced by Zn^{2+} and Hg^{2+}. The enzyme was highly specific for gallate and protocatechuate esters and showed no catalytic activity against other phenolic esters. The protein TanSg1 hydrolyzes efficiently tannic acid, a complex and polymeric gallotanin, allowing its complete conversion to gallic acid, a potent antioxidant. From its biochemical properties, TanSg1 is a tannase with potential industrial interest regarding the biodegradation of tannin waste or its bioconversion into biologically active products.

Keywords Tannase · *Streptococcus* · Hydrolase · Esterase · Gallic acid · Antioxidant

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Introduction

Tannins are water-soluble phenolic secondary metabolites of higher plants. Tannins are the fourth most abundant plant constituent, after cellulose, hemicellulose, and lignin (Lekha and Lonsane 1997). Depending on the origin of tannins, their chemistry varies widely, having a molar mass ranging from 300 to 3,000 Da, although molecules as large as 20,000 Da have been found. High tannin concentrations are found in nearly every part of the plant, such as the bark, wood, leaf, fruit, root, and seed (Serrano et al. 2009). Tannins widely occur in common foodstuffs such as tea, strawberry, raspberry, blackberry, grape, mango, cashew nut, hazelnut, walnut, and so on (Mingshu et al. 2006).

The ability of tannins to bind to proteins and other molecules causes serious environmental pollution. This implies the need for ecologically friendly degradation methods for tannic compounds. Furthermore, there is a need in the food industry to solve problems related to the binding of tannins to proteins, starch, and some other nutrients in livestock feeds since it not only affects the nutritional quality of the feed but also decreases digestibility (Chávez-González et al. 2012).

Tannins are generally resistant to biodegradation. Though tannins have toxic effects on various organisms, some microorganisms are resistant to tannins and have the ability to degrade them by the action of a tannase enzyme. Tannase, commonly referred as tannin acyl hydrolase (EC 3.1.1.20), is the most studied enzyme in the biodegradation of tannins. Tannases have received more attention because of their broad range of applications (Chávez-González et al. 2012). Tannase is widely used in the leather, pharmaceutical, beverage, and food industries. So far, the main applications of tannase are instant tea, acorn liquor, as well as gallic acid production from plant materials rich in gallotannins. In the food industry,

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tannase is used as a clarifying agent in juices and flavored coffee soft drinks; in addition, tannase helps to reduce the adverse effects of tannins in beverages and foods (Belmares et al. 2004). The main product of tannase is gallic acid. Gallic acid is used in the pharmaceutical industry as an important intermediate compound in the synthesis of trimethoprim. It is used in the chemical industry as a substrate for chemical or enzymatic synthesis of propyl gallate and other gallic acid esters, cosmetics, hair products, adhesives, and lubricants. Gallic acid is used also in the fabrication of semiconductors, dyes, and in photographic revelation. Several studies have found that gallic acid and related compounds have important therapeutic properties (Aguilar and Gutiérrez-Sánchez 2001; 2007).

Although several fungal tannases have been studied and characterized so far, the diversity of applications and conditions in which these enzymes must work also requires a large number of different enzymes capable of acting in each condition. Exploration of microbial diversity may help to find new enzymes with interesting properties (Aguilar et al. 2007; Chávez-González et al. 2012).

While many tannases from different fungi have been studied, at present, very little is known about these enzymes in bacteria. Although several sequences from the bacteria, which probably code for different tannases, have been annotated in the databases, there have been very few studies about them (Banerjee et al. 2012). Indeed such studies only concern on three bacterial tannases. A tannase from *Staphylococcus lugdunensis* was genetically identified but was not biochemically characterized (Noguchi et al. 2007). A truncated tannase from *Enterobacter* sp. has been overexpressed in *Escherichia coli* and its optimal temperature and pH were determined (Sharma and John 2011). Finally, *Lactobacillus plantarum* tannase is the only bacterial tannase that has been biochemically characterized so far, and its substrate specificity determined (Curiel et al. 2009).

In the present study, we report the cloning and expression in *E. coli* of the gene encoding TanSg1 tannase from *Streptococcus gallolyticus* UCN34. The production, purification, and biochemical characterization of the recombinant TanSg1 enzyme are also described.

Materials and methods

Bacterial strains and growth conditions

S. gallolyticus UCN34 (CIP 110142) used through this study was kindly provided by Dr. Philippe Glaser (Institut Pasteur, France). *E. coli* DH10B and *E. coli* BL21 (DE3) were used as transformation and expression hosts in the pURI3-Cter vector (Curiel et al. 2011). The *S. gallolyticus* strain was grown in BHI medium at 37 °C under static condition, and the *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 °C and shaking at 200 rpm.

PCR amplification and cloning of *S. gallolyticus* GALLO_1609 gene

Standard molecular biology techniques were performed as described by Sambrook et al. (1989). Chromosomal DNA was extracted from *S. gallolyticus* UCN34. The gene encoding for a putative tannase (*GALLO_1609*, or *tanSg1*) in *S. gallolyticus* UCN34 was PCR-amplified by Prime Star HS DNA polymerase (TaKaRa) by using the primers 774 (5′-*TAACTTTAAGAAGGAGATATACATatg*tcgattaatcaatggatttttg) and 775 (5′-*GCTATTAATGATGATGATGATGATGA TG*aacaatggcatccacccattg) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the GALLO_1609 gene sequence are written in lowercase letters). The amplification was for 30 cycles with the following conditions: 95 °C 10 s, 55 °C 5 s, and 72 °C for 1:30 min. The 1.4-kb purified PCR product was inserted into the pURI3-Cter vector using a restriction enzyme- and ligation-free cloning strategy (Curiel et al. 2011). The vector produce recombinant proteins having a six-histidine affinity tag in their C-termini. *E. coli* DH10B chemically competent cells were transformed, recombinant plasmids were isolated, and those containing the correct insert were identified by restriction enzyme analysis and verified by DNA sequencing.

The comparison of DNA and protein sequences, as well as the conceptual translation of the DNA sequence of the *tanSg1* gene, was carried out with the Basic Local Alignment Search Tool (BLAST) program in the NCBI database. Protein sequence alignments were performed using the Clustal W2 program in EMBL-EBI, and protein analysis was carried out in ExPASy (Swiss Institute of Bioinformatics).

Protein expression and purification of recombinant TanSg1 (GALLO_1609) tannase

Protein expression of the *tanSg1* gene was made using *E. coli* BL21 (DE3) cells as host strain. Cells carrying the recombinant plasmid, pURI3-Cter-TanSg1, were grown at 37 °C in LB media containing ampicillin (100 μg/ml) until an optical density at 600 nm of 0.4 was reached and then induced by adding isopropyl-β-D-thiogalactoside (IPTG) at 0.4 mM final concentration. Following induction, the cells were grown at 22 °C for 20 h and collected by centrifugation (8,000*g*, 15 min, 4 °C). The cells were resuspended in phosphate buffer (50 mM, pH 6.5). Crude extracts were prepared by French press lysis of the cell suspension (three times at 1,100 psi). The insoluble fraction of the lysate was removed by centrifugation at 47,000*g* for 30 min at 4 °C, and the supernatant was filtered through a 0.2-μm pore-size

filter and then applied to a Talon Superflow resin (Clontech) equilibrated in phosphate buffer (50 mM, pH 6.5) containing 0.3 M NaCl and 10 mM imidazole to improve the interaction specificity in the affinity chromatography step. The bound enzyme was eluted using 150 mM imidazole in the same buffer. The purity of the enzyme was determined by SDS-PAGE in Tris-glycine buffer. Fractions containing the His6-tagged protein were pooled and analyzed for tannase activity.

Enzyme activity

Colorimetric rhodanine assay for tannase activity

Tannase activity was determined using a rhodanine assay specific for gallic acid (Inoue and Hagerman 1988). Rhodanine reacts only with gallic acid and not with galloyl esters or other phenolics. Gallic acid analysis in the reactions was determined using the following assay: Tannase enzyme (100 μg) in 700 μl of 50 mM phosphate buffer pH 6.5 was incubated with 40 μl of 25 mM methyl gallate (1 mM final concentration) during 5 min at 37 °C. After this incubation, 150 μl of a methanolic rhodanine solution (0.667 %*w*/*v* rhodanine in 100 % methanol) was added to the mixture. After 5 min of incubation at 30 °C, 100 μl of 500 mM KOH was added. After an additional incubation of 5–10 min, the absorbance at 520 nm was measured on a spectrophotometer. A standard curve using gallic acid concentration ranging from 0.125 to 1 mM was prepared. One unit of tannase activity was defined as the amount of enzyme required to release 1 μmol of gallic acid per minute under standard reaction conditions.

Determination of pH and temperature profile of TanSg1

The effects of pH and temperature on the tannase activity of TanSg1 were studied by using buffers of different pH values ranging from 3.0 to 10.0. The buffers (100 mM) used were acetic acid-sodium acetate (pH 3.0–5.0), citric acid-sodium citrate (pH 6), sodium phosphate (pH 7), Tris-HCl (pH 8), glycine-NaOH (pH 9), and sodium carbonate-bicarbonate (pH 10). The rhodanine assay was used for the optimal pH characterization of tannase. Since the rhodanine-gallic acid complex forms only in basic conditions, after the completion of the enzymatic degradation of methyl gallate, KOH was added to the reaction mixture to ensure that the same pH value (pH 11) was achieved in all samples assayed.

The optimal temperature was assayed by incubating the purified TanSg1 in 50 mM phosphate buffer (pH 6.5) at different temperatures (4, 22, 30, 37, 45, 55, and 65 °C). For temperature stability measurements, the recombinant tannase was incubated in 50 mM phosphate buffer pH 6.5 at 22, 30, 37, 45, 55, and 65 °C for 30 min and 2, 4, 6, and 18 h. Aliquots were withdrawn at these incubation times to test the remaining activity at standard conditions. The nonincubated enzyme was considered as control (100 %).

Effect of additives on TanSg1

The effect of chemical inhibitors and stimulators on TanSg1 activity was investigated by the rhodanine assay using methyl gallate as substrate. The residual tannase activity was measured after the incubation of the purified enzyme with each additive. The additives analyzed were $MgCl_2$, KCl, $CaCl_2$, $HgCl_2$, $ZnCl_2$, Triton X-100, urea, Tween-80, EDTA, DMSO, and β-mercaptoethanol. The activity was expressed as a percentage of the activity level in the absence of additives. Tannase activity measured in the absence of any additive was taken as control (100 %).

Substrate specificity of TanSg1 analyzed by HPLC-DAD

The substrate specificity of TanSg1 was determined using 17 commercial phenolic esters (methyl gallate, ethyl gallate, propyl gallate, lauryl gallate, methyl benzoate, ethyl benzoate, methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate, butyl 4-hydroxybenzoate, methyl vanillate, methyl 2, 4-dihydroxybenzoate, methyl gentisate, methyl salicylate, ethyl 3,4-dihydroxybenzoate, ferulic methyl ester, and ferulic ethyl ester) as well as a natural hydrolyzable tannin (tannic acid).

The standard enzyme assay was modified by using 100 μg of TanSg1, 1 mM substrate, and 1 mM $CaCl_2$ in the reaction mixture. As controls, phosphate buffer containing the reagents but lacking the enzyme was incubated in the same conditions. The reaction products were extracted twice with ethyl acetate (Lab-Scan, Ireland) and analyzed by HPLC-DAD. A Thermo (Thermo Electron Corporation, Waltham, MA, USA) chromatograph equipped with a P4000 SpectraSystem pump and AS3000 autosampler and a UV6000LP photodiode array detector were used. A gradient of solvent A (water/acetic acid, 98:2, *v*/*v*) and solvent B (water/acetonitrile/acetic acid, 78:20:2, *v*/*v*/*v*) was applied to a reversed-phase Nova-pack C_{18} (25 cm×4.0 mm i.d.) 4.6 μm particle size, cartridge at room temperature as follows: 0–55 min, 80 % B linear, 1.1 ml/min; 55–57 min, 90 % B linear, 1.2 ml/min; 57–70 min, 90 % B isocratic, 1.2 ml/min; 70–80 min, 95 % B linear, 1.2 ml/min; 80–90 min, 100 % linear, 1.2 ml/min; 100–120 min, washing 1.0 ml/min; and reequilibration of the column under initial gradient conditions. Detection was performed by scanning from 220 to 380 nm. Samples were injected onto the cartridge after being filtered through a 0.45-μm PVDF filter. The identification of degradation compounds was carried out by comparing the retention times and spectral data of each peak with those of standards from commercial suppliers.

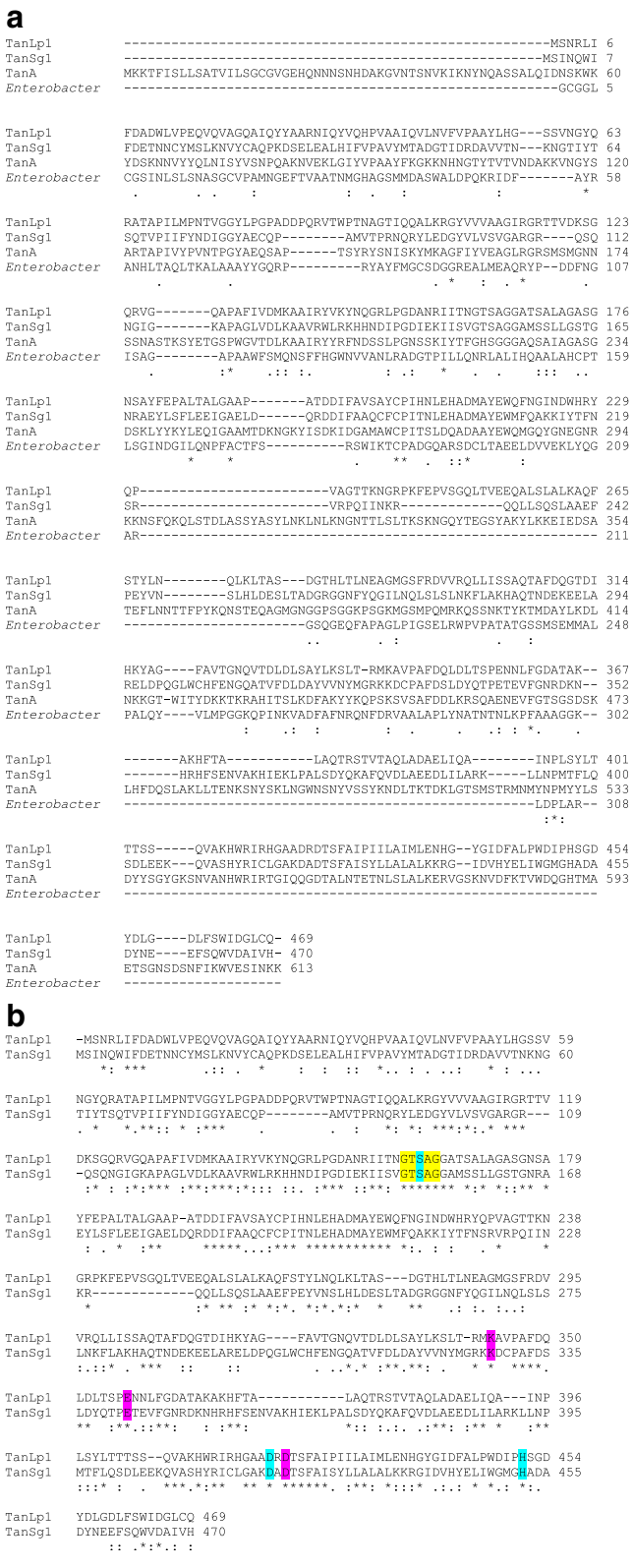

Fig. 1 Comparison of amino acid sequences of the bacterial tannases genetically identified so far. **a** Alignment of TanLp1 from *L. plantarum*, TanA from *S. lugdunensis*, and TanSg1 from *S. gallolyticus*. The amino acid sequence of the truncated tannase from *Enterobacter* sp. KPJ03 is also included. **b** Alignment of TanLp1 from *L. plantarum* and TanSg1 from *S. gallolyticus*. Multiple alignments were done using the program ClustalW2 after retrieval of sequences from BLAST homology searches. Residues that are identical (*asterisk*), conserved (*colon*), or semiconserved (*dot*) in all sequences are indicated. *Dashes* indicated gaps introduced to maximize similarities. The serine hydrolase conserved motif is highlighted in *yellow*; residues of the catalytic triad identified in the structure of TanLp1 are highlighted in *blue*; and residues which make contact with the three hydroxyl groups of gallic acid are highlighted in *pink color*

Results

Sequence comparison of TanSg1 with bacterial tannases

Osawa and Walsh (1993) demonstrated that *S. gallolyticus* strains produce an enzyme, tannase, which hydrolyzes tannins to release gallic acid. In the *S. gallolyticus* UCN34 complete genome, the TanSg1 (GALLO_1609, GenBank accession YP_003431024) protein was annotated as tannase. The DNA sequence was predicted to encode a 470-amino acid sequence protein. Calculated from the amino acid sequence, the molecular mass is 52.98 kDa and the isoelectric point (p*I*) is 5.09. The BLAST analytical program was used to compare the TanSg1 protein from *S. gallolyticus* UCN34 with those deposited in the database. TanSg1 protein is only 29 and 32 % identical to TanA from *S. lugdunensis* (GenBank accession BAF03594) and TanLp1 from *L. plantarum* (GenBank accession BAH20446), respectively, two of the three bacterial tannases genetically characterized so far (Fig. 1). It should be noted that TanA and TanLp1 are only 27 % identical among them. The third bacterial tannase genetically identified, a protein from *Enterobacter* sp. KPJ03, is only 12, 7, and 4 % identical to TanLp1 from *L. plantarum*, TanA from *S. lugdunensis*, and TanSg1 from *S. gallolyticus*, respectively. Protein searches on the databases have revealed that this protein from *Enterobacter* is similar (74 % identical) to a central region of proteins from *Klebsiella* KTE92 and *Pantoea* sp. strain AT-9b, among others (data not shown). As compared to *Klebsiella* and *Pantoea* proteins, the 308-amino acid residue protein from *Enterobacter* lacks an N-terminal region of 136 residues and a C-terminal region of 157 residues.

In spite of the low identity displayed by TanSg1 with TanA and TanLp1, this protein was selected for further study of tannase activity. The comparison of amino acid sequence of TanSg1 with TanLp1, whose tridimensional structure have been recently solved (Ren et al. 2013), revealed that the residues important for activity are conserved. TanSg1 possesses the conserved motif Gly_{150}-X-Ser-X-Gly_{154} typical of serine hydrolases. The catalytic triad identified in the TanLp1 structure is conserved in TanSg1 (Ser_{152}, Asp_{420}, and His_{452}) as well as the residues which contact with the three hydroxyl groups of gallic acid (Lys_{328}, Glu_{342}, and Asp_{422}) (Fig. 1).

Therefore, structural data suggest that TanSg1 could be an active tannase.

Production and characterization of purified recombinant TanSg1

The *tanSg1* gene was cloned into the pURI3-Cter expression vector by a ligation-free cloning strategy described previously (Curiel et al. 2011). The vector incorporates the DNA sequence encoding hexa-histidine to create a His-tagged fusion enzyme for further purification step. The integrity of the construct was confirmed by DNA sequencing.

The *tanSg1* gene was expressed in *E. coli* under the control of an IPTG-inducible promoter. Cell extracts were used to detect the presence of overproduced proteins by SDS-PAGE analysis. Whereas control cells containing the pURI3-Cter vector did not show protein overexpression, an overproduced protein with an apparent molecular mass around 53 kDa was apparent with cells harboring pURI3-Cter-TanSg1 (Fig. 2). Since the cloning strategy would yield a His-tagged protein variant, *S. gallolyticus* pURI3-Cter-TanSg1 could be purified on an immobilized metal affinity chromatography (IMAC) resin. The recombinant protein was eluted from the resin at 150 mM imidazole and observed as a single band on 10 % SDS-PAGE (Fig. 2). The enzyme was produced soluble after induction with IPTG for 18 h. Routinely, about 12 mg of purified protein from a 1-l culture was obtained.

The TanSg1 protein purified by the affinity resin was biochemically characterized. Since tannase catalyzes the hydrolysis of the galloyl ester linkage liberating gallic acid, the activity of tannase could be measured by estimating the gallic acid formed due to enzyme action (Mueller-Harvey 2001). A specific method for the detection of gallic acid could be used for a reliable quantification of tannase activity. Inoue and Hagerman (1988) described a rhodanine assay for determining free gallic acid. Rhodanine reacts only with gallic acid and not with galloyl esters or other phenolics. Rhodanine reacts with the vicinal hydroxyl groups of gallic acid to give a red complex with a maximum absorbance at 520 nm. Using methyl gallate as substrate, the specific activity of TanSg1-purified enzyme was 577 U/mg.

The optimum pH for the recombinant TanSg1 enzyme was measured at 30 °C in 50 mM phosphate buffer at different pH values. The enzyme was active between pH 4 and 9, with an optimal pH around 7, being also highly active at pH 6–8 (Fig. 3a). At pH 5, TanSg1 and TanL1 showed less than 20 % activity. At pH 3 and 10, the enzyme completely lost its activity. The optimum temperature for the recombinant enzyme (Fig. 3b) was determined in 50 mM phosphate buffer

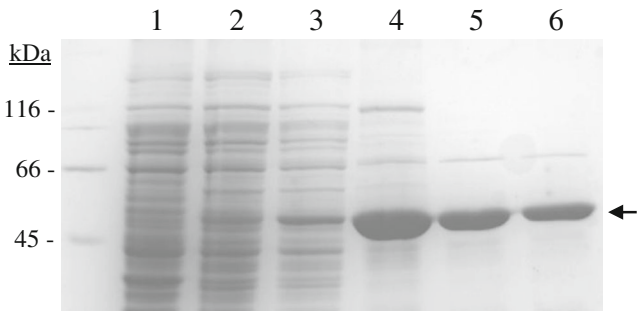

Fig. 2 Purification of recombinant *S. gallolyticus* TanSg1 protein. SDS-PAGE analysis of the expression and purification of the His_6-TanSg1. Analysis of soluble cell extracts of IPTG-induced *E. coli* BL21(DE3) (pURI3-Cter) (*1*) or *E. coli* BL21(DE3) (pURI3-Cter-TanSg1) (*2*), flowtrough (*3*), or fractions eluted after His affinity resin (*4–6*). The *arrow* indicated the overproduced and purified protein. The gel was stained with Coomassie blue. Molecular mass markers are located at the *left* (SDS-PAGE standards, Bio-Rad)

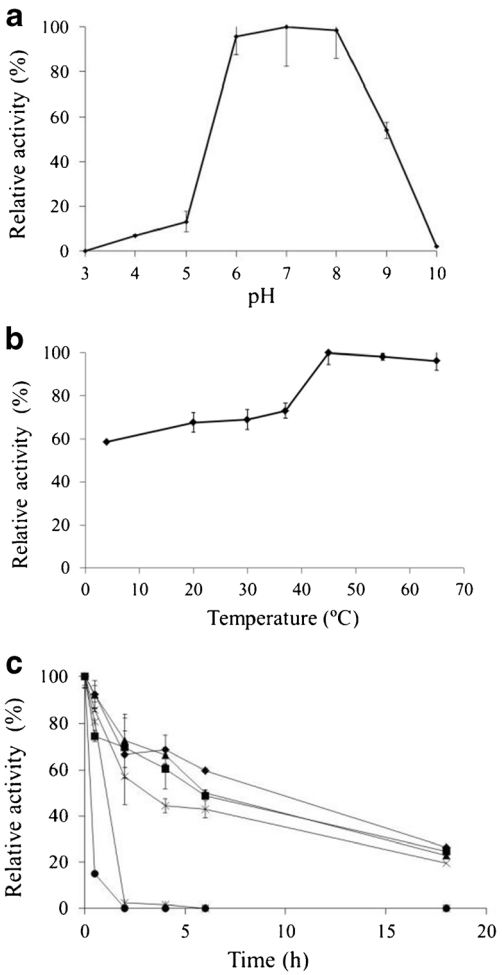

Fig. 3 Some biochemical properties of recombinant TanSg1 protein. **a** Relative activity of TanSg1 versus pH. **b** Relative activity versus temperature. **c** Thermal stability of TanSg1 after preincubation at 22 °C (*filled diamond*), 30 °C (*filled square*), 37 °C (*filled triangle*), 45 °C (*cross*), 55 °C (*star*), and 65 °C (*filled circle*) in phosphate buffer (50 mM, pH 6.5); at indicated times, aliquots were withdrawn and analyzed as described in the "Materials and methods" section. The experiments were done in triplicate. The mean value and the standard error are shown. The observed maximum activity was defined as 100 %

at pH 6.5. The protein was active between 4 and 65 °C, with 45 °C as the optimum temperature, while 60 and 90 % of the maximal activity was shown at 4 and 65 °C, respectively. At any of the temperatures assayed, the enzyme showed less than 50 % of the maximal activity. Regarding stability, the protein dramatically lost its activity after 30 min at 65 °C (Fig. 3c). However, the enzyme kept more than 70 % activity after 30 min of incubation at 55 °C. Surprisingly, the enzyme showed about 50 and 25 % of the maximal activity after 6 and 18 h of incubation at 45 °C, respectively (Fig. 3c).

The enzymatic activity of TanSg1 was tested in the presence of various metal ions and additives (Table 1). TanSg1 was activated by Ca^{2+}. DMSO and Tween-80 also activated TanSg1 protein. Enzyme activity was moderately inhibited by EDTA, Triton X-100, K^{+}, and Mg^{2+} and strongly inhibited by urea, β-mercaptoethanol, Zn^{2+}, and Hg^{2+}.

Substrate specificity of TanSg1 tannase

In order to known the substrate specificity of TanSg1, esters from different phenolic acids were assayed. The reaction products released by TanSg1 action were analyzed by HPLC-DAD. As shown in Fig. 4, from the phenolic esters assayed, TanSg1 hydrolyzed only esters from protocatechuic acid (3,4-dihydroxybenzoic acid) and gallic acid (3,4,5-trihydroxybenzoic acid). It is noteworthy to mention that only esters with a short aliphatic alcohol were effectively hydrolyzed. Methyl (C1) and ethyl (C2) gallate were efficiently hydrolyzed, whereas propyl gallate (C3) was only minimally hydrolyzed, and lauryl gallate (C12) was not hydrolyzed by TanSg1 (Fig. 4).

A hydrolysis experiment with a complex and natural tannin was carried out to determine the hydrolytic activity of TanSg1. Tannic acid, almost exclusively formed by poly-galloyl glucose derivatives, was used as natural tannin to assay TanSg1 activity. The HPLC analysis of the reaction products shown in Fig. 5 verified that TanSg1 is a true tannase. Tannic acid was fully hydrolyzed by TanSg1, and gallic acid was identified as the final product resulting from the degradation (Fig. 5). The extent of autohydrolysis without the enzyme was minimal under the conditions used (Fig. 5).

Table 1 Effect of additives on recombinant *S. gallolyticus* TanSg1 tannase activity

Additions (1 mM)	**Relative activity** (%)
Control	100
EDTA	80
KCl	69
$HgCl_2$	6
$CaCl_2$	120
$MgCl_2$	65
$ZnCl_2$	10
Triton X-100	78
DMSO	144
Tween-80	119
Urea	35
β-Mercaptoethanol	16

Discussion

Tannase has been the subject of many studies due to its commercial importance and complexity as a catalytic molecule (Chávez-González et al. 2012). Tannases are capable of hydrolyzing complex tannins, which represent the main chemical group of natural antimicrobials occurring in the plants. Tannases catalyze the hydrolysis reaction of the ester bonds present in the gallotannins, complex tannins, and gallic acid esters. The enzyme is used in food and beverage processing; however, the practical use of this enzyme is at present limited due to insufficient knowledge about its properties, optimal expression, and large-scale purification (Aguilar et al. 2007). In the past 20 years, significant progress has been made to improve production processes, including the isolation of new strains of tannase-producing microorganisms, the use of different fermentation systems, and new cost-effective purification methods. The current trend in this field is to apply molecular biology techniques to increase yields and reduce production costs. Tannase is currently commercialized by few companies that sell fungal tannase preparations with different purity and catalytic units depending on the presentation of the product (Chávez-González et al. 2012).

The diversity of applications and conditions in which tannases must work also requires a large number of different enzymes capable of acting in each condition. Exploration of microbial diversity may help to find new enzymes with interesting properties (Aguilar et al. 2007; Chávez-González et al. 2012). Proteins encoding putative tannase sequences of 149 bacteria and 36 fungi were retrieved from the NCBI database; among them, only 77 bacterial and 31 fungal putative tannase sequences were taken which have different amino acid compositions (Banerjee et al. 2012). Among the bacterial proteins, a *S. gallolyticus* protein was included. *S. gallolyticus* strains have been isolated as tannin-resistant bacteria from the feces of different mammalian herbivores, including the koala or the Japanese large wood mouse, and it is also a normal inhabitant of the rumen (Rusniok et al. 2010). Its resistance to tannins is linked to its tannase activity, a characteristic which also led this bacterium to be named *gallolyticus* as it is able to decarboxylate gallate, an organic acid derived from tannin degradation (Rusniok et al. 2010; Chamkha et al. 2002; Osawa et al. 1995; Sly et al. 1997). The identification in the *S. gallolyticus* UCN34 genome of a protein, TanSg1, similar to two bacterial tannases genetically characterized, TanA from

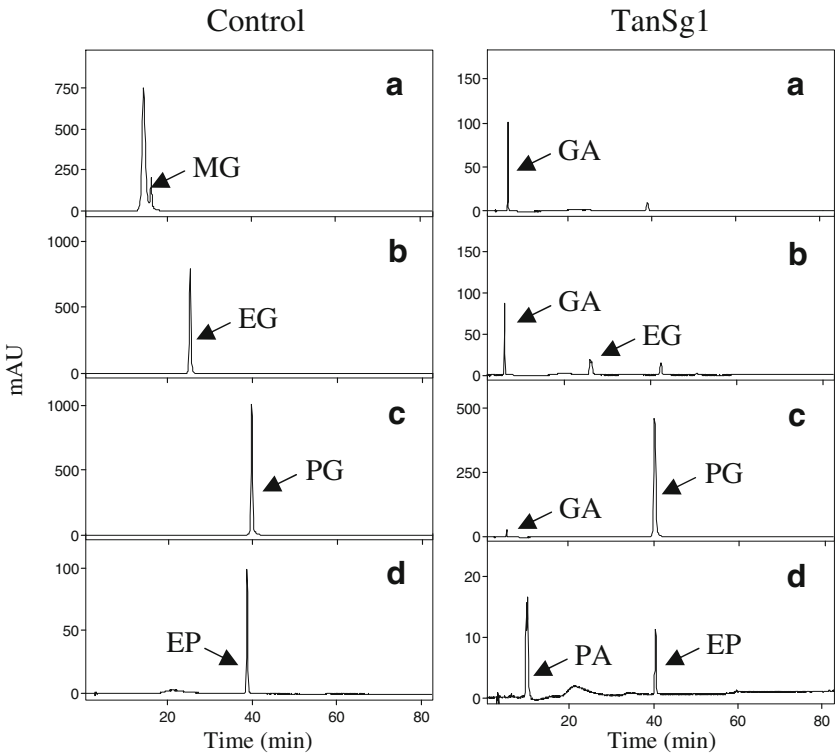

Fig. 4 Enzymatic activity of recombinant *S. gallolyticus* TanSg1 protein against commercial phenolic esters. Hydrolase activity of purified TanSg1 protein compared with control reactions on which the enzyme was omitted. HPLC chromatograms of TanSg1 (100 μg) incubated in 50 mM phosphate buffer pH 6, 1 mM $CaCl_2$, and 1 mM of methyl gallate (*A*), ethyl gallate (*B*), propyl gallate (*C*), ethyl protocatechuate (*D*). The methyl gallate (*MG*), ethyl gallate (*EG*), propyl gallate (*PG*), ethyl protocatechuate (*EP*), gallic acid (*GA*), and protocatechuic acid (*PA*) detected are indicated. The chromatograms were recorded at 280 nm

S. lugdunensis and TanLp1 from *L. plantarum*, indicated that probably it is a functional tannase.

Despite the low amino acid identity (32 %) found among TanSg1 and TanLp1 proteins, a careful examination revealed that residues important for activity are conserved. TanSg1 possesses the conserved motif typical of serine hydrolases, the catalytic triad, and the residues which contact with the three hydroxyl groups of gallic acid (Ren et al. 2013). Therefore, structural data also suggested that TanSg1 could be an active tannase.

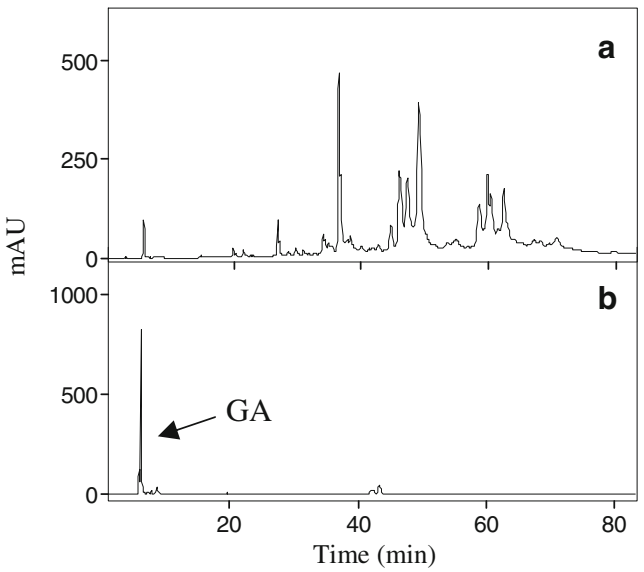

Fig. 5 Tannic acid hydrolysis by TanSg1 activity. Hydrolase activity of purified recombinant TanSg1 protein compared with control reactions on which the enzyme was omitted. HPLC chromatograms of control reaction (**a**) or TanSg1 (100 μg) (**b**) incubated in 50 mM phosphate buffer pH 6, 1 mM $CaCl_2$, and 1 mM of tannic acid. The gallic acid (*GA*) detected is indicated. The chromatograms were recorded at 280 nm

Multiple sequence alignment of fungal and bacterial tannase protein sequences showed conserved regions at different stretches with maximum homology (Banerjee et al. 2012). A phylogenetic tree showed two different clusters: one has only bacterial tannases and another has both fungi and bacteria showing some relationship between these different groups. TanLp1 from *L. plantarum* is included in the first cluster where only bacterial proteins are included. It has been described that TanLp1 represents a novel family of tannases showing no significant sequence similarity to fungal tannases (Iwamoto et al. 2008; Ren et al. 2013; Banerjee et al. 2012). TanSg1 seems to belong to the same tannase family to that of TanLp1.

The tannase activity of the recombinant TanSg1 produced in *E. coli* was well demonstrated by its ability to hydrolyze methyl gallate, an ester of gallic acid. Since tannase catalyzes the hydrolysis of the galloyl ester linkage liberating gallic acid, the activity of tannase could be measured by estimating the gallic acid formed due to enzyme action (Mueller-Harvey 2001). Inoue and Hagerman (1988) described a rhodanine assay for determining free gallic acid. Rhodanine assay was used to determine the specific activity of TanSg1; simultaneously, the activity of the previously described TanLp1 was also determined as reference. Using methyl gallate as substrate,

the specific activity was 577 U/mg for TanSg1-purified enzyme, 41 % higher than that of TanLp1 (408 U/mg). As the specific activity reported for the truncated tannase from *Enterobacter*, 13.63 U/mg (Sharma and John 2011), is significantly lower, TanSg1 exhibited the highest specific activity reported so far for a bacterial tannase.

The colorimetric rhodanine assay was used to study the biochemical properties of recombinant TanSg1 tannase. All the fungal tannase studied showed maximum activity at acidic pH values (4.3–6.5), with isoelectric points ranging from 4.3 to 5.1 in most of the cases (Chávez-González et al. 2012). The isoelectric point of TanSg1 is 5.09, similar to fungal tannases; however, the optimum pH is around 7, being also highly active at pH 6–8. Despite that TanLp1 and TanSg1 are only 32 % identical in their amino acid sequence, they showed an identical pH activity pattern. At pH 5, TanSg1 and TanL1 showed less than 20 % activity. The neutral optimum pH of bacterial tannases contrasts with the pH dependence of fungal tannases, which are acidic proteins with an optimum pH around 5.5 (Lekha and Lonsane 1997).

Most of the tannases have been reported to have optimal temperature of activity between 30 and 40 °C (Chávez-González et al. 2012). Among bacterial tannases, in contrast to TanLp1 which showed maximum activity at temperatures around 40 °C without a clear optimum (Curiel et al. 2009), TanSg1 exhibited very high activity at all temperatures assayed, reaching a maximum at 50 °C. At 4 °C, TanSg1 still displayed 60 % of the maximum activity. Likewise, 90 % of the maximum activity of TanSg1 could be observed at 65 °C. In addition, TanSg1 kept approximately half of the maximum activity after 6 h of incubation at 45 °C and 40 % after incubation at 22, 30, or 37 °C during 18 h. The high TanSg1 activity observed within this broad range of temperatures, together with its high specific activity, makes this protein the best bacterial tannase candidate for various industrial applications. Note that thermophilicity is related to the capacity of the enzyme to hydrolyze the substrate at high temperatures, while thermal stability is defined as an enzyme ability to resist thermal unfolding in the absence of its substrate. Enzymes displaying optimum activity and thermal stability at higher temperatures are attractive for biotechnological purposes in various industrial sectors. A series of competitive advantages such as faster reaction rates, decreased viscosity in processing fluids, increased solubility of the substrate, and reduced contamination risk by undesired organisms have been proposed for use of thermostable enzymes in biotechnological processes. TanSg1 characterization demonstrated that the enzyme exhibited high thermal stability under prolonged incubation up to 45 °C.

It has been described that TanLp1 from *L. plantarum* represents a novel family of tannases showing no significant sequence similarity to fungal tannases (Iwamoto et al. 2008; Ren et al. 2013). However, the reported substrate spectrum of fungal tannases and TanLp1 is similar (Curiel et al. 2009). In order to know the substrate specificity of TanSg1, esters from different phenolic acids were assayed. From the phenolic esters assayed, TanSg1 hydrolyzed only esters from protocatechuic acid (3,4-dihydroxybenzoic acid) and gallic acid (3,4,5-trihydroxybenzoic acid), a behavior that resembles that of TanLp1. However, in contrast to TanLp1, only esters with a short aliphatic alcohol were effectively hydrolyzed. Whereas TanLp1 was able to hydrolyze esters having an alcohol substituent as long as lauryl, propyl gallate was only minimally hydrolyzed by TanSg1 (Fig. 4). Structural differences among both bacterial tannases will be responsible for the different spatial requirements observed for tannase activity among both enzymes.

Since esters having a long aliphatic alcohol chain were not hydrolyzed by TanSg1, complex and natural tannins need to be studied as substrates for tannase activity. Because natural tannin extracts probably contained a range of condensed and hydrolyzable phenolic residues, it is difficult to define the substrate range of the enzyme precisely. For this reason, this study was focused on tannic acid, as a relatively well-defined commercially available hydrolyzable tannin preparation. Tannic acid obtained from oak gall nuts from *Quercus infectoria* was assayed as a tannin of natural origin. Tannic acid is almost exclusively formed by poly-galloyl glucose derivatives whose nature and complexity vary with the plant source. Cantos et al. (2003) distinguished 32 different phenolic compounds from the acorns of *Quercus* species. All of them were gallic acid derivatives. The differences encountered among these tannic acids can be attributed to the fact that different plant varieties produce different types and quantities of phenolic compounds (Hakkinen and Torronen 2000). Gallic acid is identified as the final product resulting from the degradation of hydrolyzable tannins by TanSg1 action. This is a remarkable result in view of the antioxidant properties of gallic acid. In fact, among hydroxybenzoic acids, gallic acid is the most potent antioxidant, being 1.6- and 3.4-fold more active than protocatechuic and syringic acids, respectively (Ordoudi and Tsimidou 2006). Therefore, the use of TanSg1 may provide an efficient tool to obtain molecules with valuable activities from the degradation of complex tannins present in agricultural wastes.

The use of tannase from different microbial sources may have benefits for different areas such as food, beverage, cosmetic, and pharmaceutical industries, as well as environmental depollution. Tannase may be efficient on one substrate and not on another. For that, specific tannases are needed for specific needs. The biochemical characteristics shown by TanSg1 from *S. gallolyticus* suggests that TanSg1 is a very promising enzyme for tannin degradation. TanSg1 possesses the highest specific activity and thermal stability described for a bacterial tannase. These advantages make TanSg1 an adequate candidate for industrial applications.

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Capítulo 5

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Towards unravel the degradation of gallotannins by *Streptococcus gallolyticus*

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Abstract

Background: Herbivores have developed mechanisms to overcome adverse effects of diet tannins through the presence of tannin-resistant bacteria. Tannin degradation is an unusual characteristic among bacteria. *Streptococcus gallolyticus* is a common tannin-degrader inhabitant of the gut of herbivores where plant tannins are abundant. The biochemical pathway for tannin degradation followed by *S. gallolyticus* implies the action of tannase and gallate decarboxylase enzymes to produce pyrogallol, as final product. From these proteins, only a tannase ($TanB_{Sg}$) has been characterized so far, still remaining unknown relevant proteins involved in the degradation of tannins.

Results: In addition to $TanB_{Sg}$, genome analysis of *S. gallolyticus* subsp. *gallolyticus* strains revealed the presence of an additional protein similar to tannases, $TanA_{Sg}$ (GALLO_0933). Interestingly, this analysis also indicated that only *S. gallolyticus* strains belonging to the subspecies "*gallolyticus*" possessed tannase copies. This observation was confirmed by PCR on representative strains from different subspecies. In *S. gallolyticus* subsp. *gallolyticus* the genes encoding gallate decarboxylase are clustered together and close to $TanB_{Sg}$, however, $TanA_{Sg}$ is not located in the vicinity of other genes involved in tannin metabolism. Upon methyl gallate exposure, the expression of $TanB_{Sg}$ and gallate decarboxylase was induced, whereas $TanA_{Sg}$ expression was not affected. As $TanB_{Sg}$ has been previously characterized, in this work the tannase activity of $TanA_{Sg}$ was demonstrated in presence of phenolic acid esters. $TanA_{Sg}$ showed optimum activity at pH 6.0 and 37 ºC. As compared to the tannin-degrader *Lactobacillus plantarum* strains, *S. gallolyticus* presented several advantages for tannin degradation. Most of the *L. plantarum* strains possessed only one tannase enzyme ($TanB_{Lp}$), whereas all the *S. gallolytcius* subsp. *gallolyticus* strains analyzed possesses both $TanA_{Sg}$ and $TanB_{Sg}$ proteins. More

interestingly, *S. gallolyticus* tannases presented higher activity than their *L. plantarum* counterparts. *S. gallolyticus* subsp. *gallolyticus* strains possess two-fold higher tannase activity than most *L. plantarum* strains.

Conclusions: The specific features showed by *S. gallolyticus* subsp. *gallolyticus* in relation to tannin degradation indicated that strains from this subspecies could be considered so far the best bacterial cellular factories for tannin degradation. Moreover, recombinant *S. gallolyticus* tannases showed adequate biochemical properties to be used for the biotransformation of plant tannins.

Keywords: *Streptococcus gallolyticus*, Tannase, Gallate decarboxylase, Hydrolase, Gallotannins

Background

Tannins are widespread in the plant kingdom, and are often found in woody, lignified tissues (Serrano et al., 2009). Tannins form weak, pH-dependent and reversible associations with a range of substrates including cellulose, proteins, enzymes, etc. often making the substrate resistant to microbial attack (Mingshu et al., 2006). Several reports have suggested that the presence of tannins at less than 6% dry matter of the herbivore diet may result in improved animal performance. In contrast, detrimental effects of condensed tannins in excess of 6% dry matter include decreased growth rate and body weight gain, perturbation of mineral absorption, and inhibition of digestive enzymes (Barry and Manley, 1986). Therefore, some herbivores have developed mechanisms to overcome adverse effects of tannins, at least partly, through the presence of tannin-resistant microorganisms (Krause et al., 2005).

Streptococcus gallolyticus (formerly known as *Streptococcus bovis* biotype I) has been isolated as a tannin-resistant bacterium from diverse habitats. It is a normal inhabitant of the rumen and has been isolated from feces of koalas, kangaroos, Japanese large wood mouse, cows, horses, pigs, dogs, and guinea pigs (Osawa et al., 1995; Sly et al., 1997). The presence of *S. gallolyticus* strains in the digestive tract of herbivores may play an essential role for the host in order to assimilate the tannin-rich diets from their natural habitats. The specific catabolic capacities of *S. gallolyticus* likely provide this bacterium a selective advantage to survive in the gut of herbivores, where tannins of plant origin are abundant. Therefore, a symbiotic relationship could exist between the animal host and the bacteria to counteract the antinutritional effect of dietary tannins (Sasaki et al., 2005).

S. gallolyticus strains hydrolyzed tannic acid to release gallic acid, which was subsequently decarboxylated to pyrogallol (Chamkha et al., 2002). The proposed

biochemical pathway for the degradation of tannins by *S. gallolyticus* implies the action of a tannase and a gallate decarboxylase enzyme to decarboxylate the gallic acid formed by tannase action (Chamkha et al., 2002). Pyrogallol is formed as a final product from tannin biodegradation (Chamkha et al., 2002). This pathway is also used by *Lactobacillus plantarum* strains to degrade tannins. The *L. plantarum* genes encoding a tannase (*tanB*$_{Lp}$, formerly called *tanLp*1) (Iwamoto et al., 2008) and gallate decarboxylase (*lpdBCD*) (Jiménez et al., 2013) involved in tannin degradation have been identified. In addition, a second tannase gene (*tanB*$_{Lp}$) present in some *L. plantarum* strains has been recently described (Jiménez et al., AEM). Upon tannin induction, the expression of *tanB*$_{Lp}$ was induced, whereas *tanA*$_{Lp}$ expression was not affected (Jiménez et al., AEM). Moreover, TanA$_{Lp}$ has a specific activity ten times lower than the specific activity calculated for TanB$_{Lp}$ tannase.

Similarly to *L. plantarum*, the genome of *S. gallolyticus* UCN34 revealed unique features among streptococci related to its adaptation to the rumen environment such as its ability to degrade tannins (Rusniok et al., 2010). Tannins must be degraded by the action of a tannase enzyme (Chávez-González et al., 2012). A gene encoding a nonsecreted protein similar to TanB$_{Lp}$ (GALLO_1609) was found in the *S. gallolyticus* UCN34 genome. This protein TanB$_{Sg}$ (formerly called TanSg1) has been biochemically characterized recently (Jiménez et al, AMB). In addition, another gene, GALLO_1609, encoding a 596-amino acid long protein 43% identical to the tannase from *Staphylococcus lugdunensis* (TanA$_{Sl}$) (Noguchi et al., 2007) is present in the *S. gallolyticus* UCN34 genome. Of the genes involved in tannin degradation in *S. gallolyticus*, only the *tanB*$_{Sg}$ gene encoding a tannase has been identified so far (Jiménez et al., AMB), remaining unknown the genes encoding the gallate decarboxylase enzyme as well as a putative second tannase enzyme. In this work, *S. gallolyticus* tannase and gallate decarboxylase

encoding genes involved in tannin degradation have been identified. These results provide new relevant insights into *S. gallolyticus* tannin degradation, a rare biochemical characteristic among bacteria.

Results and discussion

Sequence analysis of *S. gallolyticus* tannase enzymes. The formerly *Streptococcus bovis* group is a large bacterial complex including different species frequently isolated from animals. In 2003, the physiological differentiation between species related to the complex and clarified their respective phylogenetic position was improved (Schlegel et al., 2003). The updated classification included three subspecies: S. *gallolyticus* subsps. *gallolyticus*, *S. gallolyticus* subsp. *pasteurianus*, and *S. gallolyticus* subsp. *macedonicus*. These can be identified according to differential biochemical reactions. Among these biochemical tests, tannase activity was only described in *S. gallolyticus* subsp. *gallolyticus* strains. Only strains from these subspecies hydrolyze methyl gallate (tannase activity) and they decarboxylate gallic acid to pyrogallol (Schlegel et al., 2003) (Figure 1).

The ability to degrade tannins is a specific attribute of *S. gallolyticus* subsp. *gallolyticus* deemed important in hostile environments, as tannins are toxic polyphenolic compounds that form strong complexes with proteins and other macromolecules. The *S. gallolyticus* subsp. *gallolyticus* UCN34 genome revealed the presence of two proteins similar to tannases, $TanA_{Sg}$ (GALLO_0933) and $TanB_{Sb}$ (GALLO_1609). The tannase proteins from *S. lugdunensis* ($TanA_{Sl}$), *L. plantarum* ($TanA_{Lp}$ and $TanB_{Lp}$) and *S. gallolyticus* ($TanA_{Sg}$ and $TanB_{Sg}$) were aligned. The alignment revealed that two protein group could be easily identified. TanA proteins, higher than 60 kDa and possessing a cleavage site of a peptide signal, and TanB proteins, having around 50 kDa of molecular size. The identity degree among TanA and TanB proteins is lower than 30%. The

131 comparison of the amino acid sequence of these tannase proteins with $TanB_{Lp}$, whose
132 tridimensional structure have been recently solved (Ren et al., 2013), revealed that the
133 residues important for activity are conserved. All the analyzed proteins possessed the
134 conserved motif Gly-X-Ser-X-Gly typical of serine hydrolases. The catalytic triad
135 identified in the $TanB_{Lp}$ structure (Ser-163, Asp-419, and His-451) is only conserved in
136 the TanB proteins. In both TanA proteins Asp-419 is substituted by a Gln residue. This
137 amino acid variation was noticed previously (Ren et al., 2013) and suggested that the
138 conserved residue Asp-421 may fulfil the role of Asp-419 as the acidic residue of the
139 catalytic triad. The residues identified that make contacts with the three hydroxyl groups
140 of gallic acid (Asp-421, Lys-343, and Glu-357, in $TanB_{Lp}$) are conserved in all the tannase
141 proteins analyzed (Figure 2A). When TanA proteins were compared identity degrees
142 ranging from 44-50% were found (Figure 2B). However, the identity among TanB
143 proteins was lower (32%) (Figure 2C).
144 As described previously for *S. gallolyticus* spp., only strains from the subspecies
145 *gallolyticus* possess tannase activity (Schlegel et al., 2003). However, it is not known if
146 both tannase proteins are present in all the *S. gallolyticus* subsps. *gallolyticus* strains.
147 Currently, the genomes of four *S. gallolyticus* subsb *gallolyticus* strains are available
148 (UCN34, ATCC 43143, ATCC BAA-2069, and TX2005 strains). A genome search
149 revealed that the four strains possessed both tannase genes, $TanA_{Sg}$ (GALLO_0933,
150 SGGB_0917, SGGBAA2069_c09070/80, and HMPREF9352_1611) or $TanB_{Sg}$
151 (GALLO_1609, SGGB_1624, SGGBAA2069_c16370, and HMPREF9352_0937) in
152 UCN34, ATCC 43143, ATCC BAA-2069, and TX2005 strains, respectively. In relation to
153 $TanA_{Sg}$ from UCN34 strain, the proteins from TX2005 and ATCC 43143 strains exhibited
154 3, or 5 amino acid changes (data not shown). In addition, the $TanA_{Sg}$ protein from ATCC
155 BAA-2069 posses a mutation at position Tyr-230 which originates a stop codon, and

therefore, a truncated protein. In relation to $TanB_{Sg}$ proteins, protein from UCN34, ATCC 43143, and ATCC BBA-2069 strains were identical, however, protein from TX2005 strain showed 20 amino acid substitutions (data not shown). These results seem to indicate that all the strains from the *S. gallolyticus* subssp. *gallolyticus* possessed both, $tanA_{Sg}$ and $tanB_{Sg}$ genes. In order to associate the presence of these genes with the "*gallolyticus*" subspecies, DNA from different *S. gallolyticus* subspecies was used to amplify $tanA_{Sg}$ and $tanB_{Sg}$ genes. From the strains assayed, oligonucleotides 803-804, and 774-775 amplified 1.7 and 1.4 kb DNA fragments from $tanA_{Sg}$ and $tanB_{Sg}$, respectively, only in *S. gallolyticus* DSM 13808, *S. gallolyticus* subsp. *gallolyticus* UCN34, and *S. gallolyticus* subsp. *gallolyticus* DSM 16831^T strains (Figure 3). No amplification was observed in strains belonging to the other *S. gallolyticus* subspecies, e.g. *S. gallolyticus* subsp. *pasteurianus* DSM 15351^T, and *S. gallolyticus* subsp. *macedonius* DSM 15879^T. These results indicated that similarly to tannase activity, the presence of $tanA_{Sg}$ and $tanB_{Sg}$ genes seems to be specific for the subspecies *gallolyticus*.

Tannin-induced gene expression of the *S. gallolyticus* region involved in tannin degradation

The presence of both tannase genes in all the *S. gallolyticus* subsp. *gallolyticus* strains analyzed is in contrast with the scarce presence of $tanA_{Lp}$ in *L. plantarum* strains. Only 4 strains out 29 *L. plantarum* strains analyzed possessed both tannase genes. Another significant difference among *L. plantarum* and *S. gallolyticus* strains is the organization of the genes involved in tannin degradation. In *L. plantarum* the genes encoding gallate decarboxylase (*lpdC*, and *lpdBD*) are separated in the chromosome by more than 1 Mb (Jiménez et al., 2013); however in *S. gallolyticus*, the genes encoding gallate decarboxylase are clustered together (*sgdCBD*, GALLO_1613, GALLO_1612, and

GALLO_1611, respectively) (Figure 4). All the *S. gallolyticus* strains whose genome is currently available, shared the same genetic organization showed by *S. gallolyticus* UCN34 strain (Figure 4). In *S. gallolyticus* strains, $TanA_{Sg}$ and $TanB_{Sg}$ are separated from 691 (TX2005 strain) to 750 kb (ATCC BAA-2069 strain). Similarly to *L. plantarum*, TanB is located close to subunit C, the catalytic subunit of the gallate decarboxylase. However, TanA is not located in the vicinity of other genes involved in the metabolism of tannins in *L. plantarum* and *S. gallolyticus*. The different chromosomal location of both tannase genes could indicate a different function and regulation.

In order to know the specific physiological role of both *S. gallolyticus* tannases the study of their synthesis under the presence of a substrate could provide relevant data. As tannase and gallate decarboxylase are involved in tannin degradation, the relative expression of their encoding-genes under methyl gallate exposure was studied. *S. gallolyticus* UCN34 cultures were induced for 10 min by the presence of 15 mM methyl gallate as potential tannase substrate. The gene expression levels obtained were substantially different among both tannase encoding genes, indicating the presence of two different expression patterns for these proteins. The $tanA_{Sg}$ gene showed an expression level not affected by the presence of its substrate methyl gallate. However, the $tanB_{Sg}$ gene expression profile was affected. The presence of 15 mM methyl gallate induces about a 3-fold increase in the expression of $tanB_{Sg}$ gene. This expression behaviour allows assuming that $tanB_{Sg}$ encodes an inducible tannase in *S. gallolyticus* subsp. *gallolytcius,* as previously observed in *L. plantarum* strains (Jiménez et al., 2014).

Biochemical properties of *S. gallolyticus* UCN34 $TanA_{Sg}$ tannase

When $TanB_{Lp}$ was the only tannase described from *L. plantarum*, homology searched allowed us to identify $tanB_{Sg}$ (GALLO_1609). The gene was cloned and expressed in *E.*

206 *coli*, and the recombinant $TanB_{Sg}$ protein was biochemically characterized (Jiménez et al.,
207 AMB). The data indicated that, as compared to $TanB_{Lp}$, $TanB_{Sg}$ possess interested
208 biochemical properties. $TanB_{Sg}$ has a specific activity 41% higher than $TanB_{Lp}$, and
209 displayed optimum activity at pH 6-8 and 50-70 ºC, showing high stability over a broad
210 range of temperatures (Jiménez et al., AMB). However, in relation to its substrate range
211 and contrarily to $TanB_{Lp}$, only esters with a short aliphatic alcohol were effectively
212 hydrolyzed by $TanBS_g$. Taking into account only the activity of TanB tannases, it seems
213 that *S. gallolyticus* could hydrolyze tannins more efficiently than *L. plantarum* strains.
214 Recently it has been described that in a few *L. plantarum* strains, a second tannase
215 gene ($tanA_{Lp}$) could be found (Jiménez et al., AEM). The $TanA_{Lp}$ protein, in relation to
216 $TanB_{Lp}$, presented ten times lower specific activity, and showed differences on its optimal
217 pH and temperature. In order to demonstrate the functionality of $TanA_{Sg}$ protein, and to
218 know its biochemical properties and relevance on tannin degradation, the $tanA_{Sg}$ gene was
219 expressed in *E. coli* under the control of an IPTG inducible promoter. Cell extracts were
220 used to detect the presence of overproduced proteins by SDS-PAGE analysis. Whereas
221 control cells containing the pURI3-TEV vector did not show protein overexpression, an
222 overproduced protein with an apparent molecular mass around 63 kDa was apparent with
223 cells harbouring pURI3-TEV-$TanA_{Sg}$ (Figure 5). Since the cloning strategy yields a His-
224 tagged protein variant, *S. gallolyticus* pURI3-TEV-$TanA_{Sg}$ could be purified on an
225 immobilized metal affinity chromatography (IMAC) resin. The recombinant protein was
226 eluted from the resin at 150 mM imidazole, and observed as single band on 10% SDS-
227 PAGE (Figure 5).
228 To demonstrate tannase activity, the $TanA_{Sg}$ protein purified by affinity
229 chromatography was incubated in the presence of different esters of gallic acid. As
230 expected for a tannase enzyme, $TanA_{Sg}$ was able to hydolyze esters from gallic acid,

231 confirming their tannase activity (Figure 6-1). $TanA_{Sg}$ hydrolyzes methyl, ethyl and propyl
232 gallate, but similarly to $TanB_{Sg}$ and $TanA_{Lp}$, was unable to hydrolyze esters having an
233 alcohol substituent as long as lauryl, which was hydrolyzed by $TanB_{Lp}$. As a general rule
234 of the bacterial tannases described so far, a protocatechuate ester (ethyl protocatechuate)
235 was also hydrolyzed by $TanA_{Sg}$ (Curiel et al., 2009). Esters from other phenolic acid
236 assayed were not hydrolyzed (data not shown). These results confirmed that $TanA_{Sg}$ is a
237 functional tannase and its substrate range resembles that of the bacterial tannases
238 described previously (Curiel et al., 2009; Jiménez et al., AMB; Jiménez et al., AEM).
239 Different dietary plant varieties produce different types and quantities of phenolic
240 compounds (Hakkinen and Torronen, 2000). As the digestive tract of herbivores probably
241 contains different tannins, we focused our studies on the relatively well-define
242 commercially available preparations, tannic acid and epigallocatechin gallate. Tannic acid
243 is almost exclusively formed by poly-galloyl glucose and gallic acid was observed as the
244 final product of $TanA_{Sg}$ activity on tannic acid (Figure 6-2). Similarly, gallic acid was
245 liberated from epigallocatechin as a result of the action of $TanA_{Sg}$ on epigallocatechin
246 gallate. These results confirmed the capacity of $TanA_{Sg}$ to degrade dietary tannins.
247 Once the tannase activity of $TanA_{Sg}$ was confirmed, their biochemical properties
248 were determined. Since tannase catalyzes the hydrolysis of the galloyl ester linkage
249 liberating gallic acid, the activity of tannase could be measured by estimating the gallic
250 acid formed due to enzyme action (Mueller-Harvey, 2001). A rhodanine specific method
251 for the detection of gallic acid was used for a reliable quantification of tannase activity.
252 (Inoue and Hagerman, 1988). Rhodanine reacts with the vicinal hydroxyl groups of gallic
253 acid to give a red complex with a maximum absorbance at 520 nm. Rhodanine assay was
254 used to determine the specific activity of $TanA_{Sg}$. Using methyl gallate as substrate, the
255 specific activity of $TanA_{Sg}$ was 256 U/mg, 55% or 37% lower than the specific activity of

256 $TanB_{Sg}$ (577 U/mg) or $TanB_{Lp}$ (408 U/mg); however, it was more than 6 times higher than
257 the activity of the equivalent protein from *L. plantarum*, $TanA_{Lp}$ (39 U/mg). These activity
258 data indicated that *S. gallolyticus* strains have more potential to degrade tannins than *L.*
259 *plantarum* strains. Firstly, in *L. plantarum* only a small number of strains possessed $tanA_{Lp}$
260 gene, whereas all the *S. gallolytius* subsp. *gallolyticus* strains analyzed so far possesses
261 both tannase genes. More important, tannases from *S. gallolyticus* possess higher specific
262 activity than their counterparts from *L. plantarum*. In a simplify sight, *S. gallolyticus*
263 having $TanA_{Sg}$ and $TanB_{Sg}$ posses a two-fold higher tannase activity than most *L.*
264 *plantarum* strains which posses only $TanB_{Lp}$ tannase.

265 In order to known if $TanA_{Sg}$ posses additional interested biochemical features, its
266 optimal pH and temperature was analyzed. The biochemical characterization of $TanA_{Sg}$
267 revealed that their optimal pH is around 6, being also highly active at pH 6-8 (Figure 7A).
268 This optimal pH is slightly lower than that reported for $TanB_{Lp}$ (optimal pH 7.0-8.0)
269 (Iwamoto et al., 2008; Curiel et al., 2009), but similar than that of $TanB_{Sg}$ and $TanA_{Lp}$.
270 The optimum temperature for activity is 37 ºC, and also exhibited very high activity at
271 higher temperatures (Figure 7B). At 65 ºC $TanA_{Sg}$ showed more than 80% of the
272 maximum activity. $TanA_{Sg}$ is able to hydrolyze the substrate (methyl gallate) at high
273 temperatures. In addition, $TanA_{Sg}$ showed thermal stability higher than that reported for
274 $TanB_{Lp}$, since it retained more than 70% of the maximal activity after 18 h incubation at
275 37 ºC (Figure 7C). Non-ionic detergents showed different effect on $TanA_{Sg}$ activity.
276 Whereas Triton-X-100 did not affect activity, Tween-80 greatly increased tannase activity
277 (Figure 7D). Similarly to $TanB_{Lp}$, among metal ions, Ca^{2+} and K^{+} increased and Hg^{2+}
278 greatly inhibited $TanA_{Sg}$ activity (Curiel et al., 2009). The ions Zn^{2+} and Mg^{2+} partially
279 inhibited the enzyme. The activity of $TanA_{Sg}$ was also significantly inhibited by β-
280 mercaptoethanol.

In addition to $TanB_{Sg}$ activity, the biochemical properties showed by $TanA_{Sg}$ such as high specific activity, optimal temperature (37 ºC) and broad pH range indicated that *S. gallolyticus* subsp. *gallolyticus* strains are so far the bacterial cells better adapted to degrade the tannins present on the diet. It has been described that *S. gallolyticus* initially responds to tannins with an increased lag phase and decreased growth rate (O´Donovan and Brooker, 2001). The response mechanism followed by *S. gallolyticus* strains enables then to maintain growth and biosynthetic capacity in the presence of high tannin concentrations.

Conclusions

In summary, this study confirmed among bacteria the specific characteristics present in *S. gallolyticus* subsp. *gallolyticus* strains which could be important for survival in the gut environment of herbivores, where a large diversity of tannins is present. *S. gallolyticus* should be able to degrade these polyphenols and it is known that this bacterium does not depend on other microorganisms for their degradation. The tannin degradation pathway followed by *S. gallolyticus* strains implies the combined action of tannase and gallate decarboxylase, which are close on *S. gallolyticus* genome and seems to act coordinately. In addition, the presence in all the *S. gallolyticus* strains of a second and extracellular tannase enzyme provide them additional advantages for the degradation of high-molecular tannins unable to pass into the cell. The specific features showed by *S. gallolyticus* subsp. *gallolyticus* in relation to tannin degradation suggested that these strains are the best bacterial factories for tannin degradation described so far. This explains the widespread occurrence of *S. gallolyticus* in the rumen of livestock that frequently browse tannin-containing forages, and it is likely that the presence of *S. gallolyticus* provides a selective advantage to these animals.

Materials and methods

Bacterial strains and growth conditions

S. gallolyticus subsp. *gallolyticus* UCN34 (CIP 110142) used through this study was kindly provided by Dr. Philippe Glaser (Institut Pasteur, France). *S. gallolyticus* DSM 13808, *S. gallolyticus* subsp. *gallolyticus* DSM 16831^T, *S. gallolyticus* subsp. *pasteurianus* DSM 15351^T, and *S. gallolyticus* subsp. *macedonius* DSM 15879^T were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). *Escherichia coli* DH10B and *E. coli* BL21 (DE3) were used as transformation and expression hosts in the pURI3-TEV vector (Curiel et al., 2011). The *S. gallolyticus* strains were grown in BHI medium at 37 ºC without shaking, and the *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 ºC and shaking at 200 rpm.

PCR detection of tannase encoding genes

Genes encoding *S. gallolyticus* tannases (*tanA*$_{Sg}$ and *tanB*$_{Sg}$) were amplified by PCR using chromosomal DNA from *S. gallolyticus* strains. The *tanA*$_{Sg}$ gene (1.7 kb) was amplified by using primers 803 and 804 (Table 1). Oligonucleotides 774 and 775 were used to amplify *tanB*$_{Sg}$ gene (1.4 kb). The reactions were performed in a Personal thermocycler (Eppendorf), using 30 cycles of denaturation at 94 ºC for 30 s, annealing at 55 ºC for 1 min, and extension at 72 ºC for 30 s. Amplified fragments were resolved in agarose gels.

Quantitative PCR. For RNA isolation, BHI cultures of *S. gallolyticus* subsp. *gallolyticus* UCN34 were grown up to an OD 600nm of 1 and then supplemented with methyl gallate at 15 mM final concentration. As control, RNA was also isolated from cultures not supplemented with methyl gallate. After 10 min incubation the cultures were immediately

processed for RNA extraction as previously described (Saulnier et al., 2007). After DNaseI treatment, the absence of DNA from the RNA samples was verified by PCR. The DNA-free RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosyntems) according to the manufacturer instructions. From the DNA obtained, quantitative gene expression was analyzed in an AbiPrism 7500 Fast Real Time PCR system (Applied Biosystems). Specific primer pairs were designed with the Primer Express 3.0 program to amplify internal regions of tannase and gallatoe decarboxylase encoding genes (Table 1). Amplifications were performed in triplicate. All qPCR assays amplified a single product as determined by melting curve analysis and by electrophoresis. A standard curve was plotted with cycle threshold (Ct) values obtained from amplification of known quantities of cDNA and used to determine the efficiency (E) as $E=10^{-1/slope}$. In order to measure *S. gallolyticus* gene expression, amplification of the endogenous control gene was performed simultaneously and its relative expression compared with that of the target gene. Relative expression levels were calculated with the 7500 Fast System relative quantification software using *S. gallolyticus ldh* gene as endogenous gene and the growth in the absence of methyl gallate as growth condition calibrator.

Cloning, experession and purification of $TanA_{Sg}$ from *S. gallolyticus* UCN34

The gene encoding a putative tannase (*GALLO_0933*, or $tanA_{Sg}$) in *S. gallolyticus* UCN34 (accession YP_003430356) was PCR-amplified by PrimeSTAR HS DNA polymerase (Takara). As a peptide signal was predicted in the $TanA_{Sg}$ sequence, primers 803 and 804 were used to amplify and clone $TanA_{Sg}$ lacking the 26-amino acid peptide signal sequence. The gene was cloned into the pURI3-TEV vector which encodes expression of a leader sequence containing a six-histidine affinity tag. The purified 1.7-kb PCR product

was then inserted into the vector by using a restriction enzyme- and ligation-free cloning strategy (Curiel et al., 2011).

E. coli DH10B cells were transformed, recombinant plasmids were isolated, and those containing the correct insert were identified by restriction enzyme analysis, verified by DNA sequencing, and then transformed into *E. coli* BL21 (DE3) cells for expression. *E. coli* BL21 (DE3) cells carrying the recombinant plasmid, pURI3-TEV-TanA_{Sg}, were grown at 37 ºC in LB media containing ampicillin (100 μg/ml) on a rotary shaker (200 rpm) until an optical density at 600 nm of 0.4 was reached. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.4 mM and protein induction was continued at 22 ºC for 18 h.

The induced cells were harvested by centrifugation (8,000 *g*, 15 min, 4 ºC), resuspended in phosphate buffer (50 mM, pH 6.5) and disrupted by French Press passages (three times at 1,100 psi). The insoluble fraction of the lysate was removed by centrifugation at 47,000 *g* for 30 min at 4 ºC. The supernatant was filtered through a 0.2 μm pore-size filter and then applied to a Talon Superflow resin (Clontech) equilibrated in phosphate buffer (50 mM, pH 6.5) containing 3 mM NaCl and 10 mM imidazole to improve the interaction specificity in the affinity chromatography step. The bound enzyme was eluted using 150 mM imidazole in the same buffer. The purity of the enzymes was determined by SDS-PAGE in Tris-glycine buffer. Fractions containing the His6-tagged protein were pooled and analyzed for tannase activity.

Tannase activity assay

Tannase activity was determined using a rhodanine assay specific for gallic acid (Inoue and Hagerman, 1988). Rhodanine reacts only with gallic acid and not with galloyl esters or other phenolics. Gallic acid analysis in the reactions was determined using the

following assay. Tannase enzyme (100 µg) in 700 µl of 50 mM phosphate buffer pH 6.5 was incubated with 40 µl of 25 mM methyl gallate (1 mM final concentration) for 5 min at 37 ºC. After this incubation, 150 µl of a methanolic rhodanine solution (0.667% w/v rhodanine in 100% methanol) were added to the mixture. After 5 min incubation at 30 ºC, 100 µl of 500 mM KOH was added. After an additional incubation of 5-10 min, the absorbance at 520 nm was measured on a spectrophotometer. A standard curve using gallic acid concentration ranging from 0.125 to 1mM was prepared. One unit of tannase activity was defined as the amount of enzyme required to release 1 µmol of gallic acid per minute under standard reaction conditions.

Biochemical properties of $TanA_{Sg}$ tannase

The pH profile of $TanA_{Sg}$ activity was determined using different 100 mM buffer systems containing acetic acid-sodium acetate (pH 3.0-5.0), citric acid-sodium citrate (pH 6), sodium phosphate (pH 7), Tris-HCl (pH 8), glycine-NaOH (pH 9), and sodium carbonate-bicarbonate (pH 10). The rhodanine assay was used for the optimal pH characterization of tannase. Since the rhodanine-gallic acid complex forms only in basic conditions, after the completion of the enzymatic degradation of methyl gallate, KOH was added to the reaction mixture to ensure that the same pH value (pH 11) was achieved in all samples assayed.

The optimal temperature for purified $TanA_{Sg}$ tannase was determined in the temperature range 4-65 ºC in 25 mM phosphate buffer (pH 6.5). For determination of the thermal stability of $TanA_{Sg}$, the enzyme in 50 mM phosphate buffer pH 6.5 was preincubated in the absence of substrate at 22, 30, 37, 45, 55 and 65 ºC for 30 min and 2, 4, 6, and 18 h. Aliquots were withdrawn at these incubation times to test the remaining

405 activity under standard conditions. The residual tannase activity was determined at 37 ºC.
406 The non-heated enzyme was considered as control (100%).
407 The enzyme was pre-incubated in the presence of various metal salts and chemical
408 agents using final concentrations of 1 mM. The effect of chemical inhibitors and
409 stimulators on $TanA_{Sg}$ activity was investigated by the rhodanine assay using methyl
410 gallate as substrate. The additives analyzed were $MgCl_2$, KCl, $CaCl_2$, $HgCl_2$, $ZnCl_2$,
411 Triton-X-100, Urea, Tween 80, EDTA, DMSO, and β-mercaptoethanol. The extent of
412 inhibition or activation of tannase activity was indicated as the percentage of the ratio of
413 residual activity to complete enzyme activity in the control sample without addition of
414 metal ions or chemical agents. Tannase activity measured in the absence of any additive
415 was used as control (100%).
416 The substrate specificity of $TanA_{Sg}$ was determined using 17 commercial phenolic
417 esters (methyl gallate, ethyl gallate, propyl gallate, lauryl gallate, methyl benzoate, ethyl
418 benzoate, methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, propyl 4-
419 hydroxybenzoate, butyl 4-hydroxybenzoate, methyl vanillate, methyl 2, 4-
420 dihydroxybenzoate, methyl gentisate, methyl salicylate, ethyl 3, 4-dihydroxybenzoate,
421 ferulic methyl ester, and ferulic ethyl ester) as well as a natural tannins (epigallocatechin
422 gallate, and tannic acid). As controls, phosphate buffer containing the reagents but lacking
423 the enzyme were incubated in the same conditions.
424 The hydrolysis products were extracted twice with ethyl acetate (Lab-Scan,
425 Ireland) and analyzed by HPLC-DAD. A Thermo (Thermo Electron Corporation,
426 Waltham, Massachussetts, USA) chromatograph equipped with a P400 SpectraSystem
427 pump, and AS3000 autosampler, and a UV6000LP photodiode array detector were used. A
428 gradient of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic
429 acid, 78:20:2, v/v/v) was applied to a reversed-phase Nova-pack C_{18} (25 cm x 4.0 mm i.d.)

4.6μm particle size, cartridge at room temperature as follows: 0-55 min, 80% B linear, 1.1 ml/min; 55-57 min, 90% B linear, 1.2 ml/min; 57-70 min, 90% B isocratic, 1.2 ml/min; 70-80 min, 95% B linear, 1.2 ml/min; 80-90 min, 100% linear, 1.2 ml/min; 100-120 min, washing 1.0 ml/min, and reequilibration of the column under initial gradient conditions. Detection was performed by scanning from 220 to 380 nm. Samples were injected onto the cartridge after being filtered through a 0.45 μm PVDF filter.

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***gallolyticus* subsp. *macedonius* subsp. nov. and *S. gallolyticus* subsps. *pasteurianus* subsp. nov**. *Int J System Evol Microbiol* 2003, **53**:631-645.

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Figure legends

Figure 1. Schematic representation of tannin degradation pathway followed by *S. gallolyticus*. When R_1 is (H) the represented compounds are a protocatechuate ester, protocatechuic acid, and catechol. When R_1 is (OH) the represented compounds are a gallate ester, gallic acid, and pyrogallol. R_2 (OH) could be (OCH_3) representing methyl ester of gallic/protocatechuic acid, (OCH_2CH_3) representing ethyl esters, ($OCH_2CH_2CH_3$) propyl esters, (glucose) as in tannic acid, or (epigallocatechin) in epigallocatechin gallate. $TanA_{Sg}$ and $TanB_{Sg}$ represented tannase enzymes and SgdB, SgdC, and SgdD, represent the three subunits of gallate decarboxylase enzyme.

Figure 2. Comparison of amino acid sequences of bacterial tannases. (A) $TanA_{Sl}$ from *Staphylococcus lugdunensis*, $TanA_{Lp}$ and $TanB_{Lp}$ from *Lactobacillus plantarum*, and $TanA_{Sg}$ and $TanB_{Sg}$ from *Streptococcus gallolyticus* subsp. *gallolyticus*. (B) Alignment of TanA or (C) TanB proteins. Multiple alignments were done using the program ClustalW2 after retrieval of sequences from BLAST homology searches. Residues that are identical (*), conserved (:) or semiconserved (.) in all sequences are indicated. Dashes indicated gaps introduced to maximize similarities. The vertical arrow indicated the predicted peptide signal cleavage site. The serine hydrolase conserved motif is highlighted in yellow; residues of the catalytic triad identified in the structure of $TanB_{Lp}$ are highlighted in blue; and residues which make contacts with the three hydroxyl groups of gallic acid are highlighted in pink color.

Figure 3. PCR amplification of tannase encoding genes from several *S. gallolyticus* strains. (A) Amplification of 1.7-kb DNA fragment of *$tanA_{Sg}$* with oligonucleotides 803-

804. (B) Amplification of 1.4-kb *tanB*$_{Sg}$ fragment with oligonucleotides 774-775. Chromosomal DNA from the following *S. gallolyticus* strains was used for PCR amplification: *S. gallolyticus* DSM 13808 (1), *S. gallolyticus* subsp. *gallolyticus* DSM 16831^T (2), *S. gallolyticus* subsp. *gallolyticus* UCN34 (3), *S. gallolyticus* subsp. *pasteurianus* DSM 15351^T (4), and *S. gallolyticus* subsp. *macedonius* DSM 15879^T (5). PCR fragments were subject to agarose gel electrophoresis and stained with Gel Red. Left lane, λ-EcoT14I digest (Takara). Numbers indicated some of the molecular sizes (in kb).

Figure 4. Genetic organization of the *S. gallolyticus* subsp. *gallolyticus* UCN34 chromosomal region containing gallate decarboxylase and tannase encoding genes (accession NC_013798, positions 1708280/c-1698323/c and 984535/c-981288/c). The genetic organization of the chromosomal region containing the same genes in *L. plantarum* WCFS1 (NC_004567) or ATCC 14917^T (ACGZ02000013.1) is also represented. Arrows indicate genes. Genes coding for TanA tannase proteins are coloured in blue and TanB in pink. Genes encoding gallate decarboxylase subunits are coloured in yellow and each different subunit is represented by different drawing.

Figure 5. **SDS-PAGE analysis of the expression and purification of *S. gallolyticus* TanA$_{Sg}$ protein**. Analysis of soluble cell extracts of IPTG-induced *E. coli* BL21(DE3) (pURI3-TEV) (1) or *E. coli* BL21(DE3) (pURI3-TEV-TanA$_{Sg}$) (2), flowtrough (3), or fractions eluted after His affinity resin (4-8). The arrow indicated the overproduced and purified protein. The gel was stained with Coomassie blue. Molecular mass markers are located at the left (SDS-PAGE Standards, Bio-Rad).

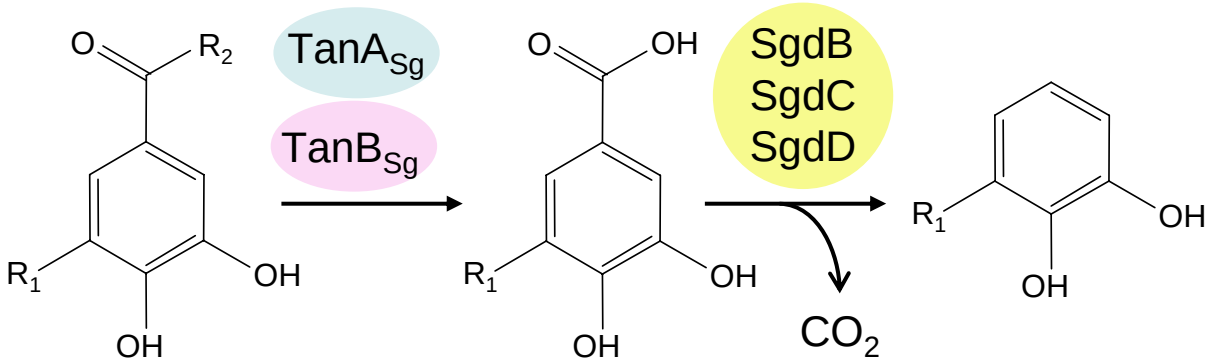
574 **Figure 6**. **Tannase activity of *S. gallolyticus* $TanA_{Sg}$ protein against simple phenolic**
575 **esters (1) and complex tannins (2)**. Hydrolase activity of purified $TanA_{Sg}$ protein
576 compared with control reactions on which the enzyme was omitted. HPLC chromatograms
577 of $TanA_{Sg}$ (100 μg) incubated in 1 mM of methyl gallate (A), ethyl gallate (B), propyl
578 gallate (C), ethyl protocatechuate (D), epigallocatechin gallate (E), and tannic acid (F).
579 The methyl gallate (MG), ethyl gallate (EG), propyl gallate (PG), ethyl protocatechuate
580 (EP), epigallocatechin gallate (EGCG), tannic acid (TA), gallic acid (GA), protocatechuic
581 acid (PA), and epigallocatechin (EGC) detected are indicated. The chromatograms were
582 recorded at 280 nm.
583
584 **Figure 7. Some biochemical properties of $TanA_{Sg}$ protein.** (A) Relative activity versus
585 pH. (B) Relative activity of $TanA_{Sg}$ versus temperature. (C) Thermal stability of $TanA_{Sg}$
586 after preincubation at 22 ºC (filled diamond), 30 ºC (filled square), 37 ºC (filled triangle),
587 45 ºC (cross), 55 ºC (star), and 65 ºC (filled circle) in phosphate buffer (50 mM, pH 6.5);
588 at indicated times, aliquots were withdrawn, and analyzed as described in the Methods
589 section. The observed maximum activity was defined as 100%. (D) Relative activity of
590 $TanA_{Sg}$ after incubation with 1mM concentrations of different additives. The activity of
591 the enzyme in the absence of additives was defined as 100%. The experiments were done
592 in triplicate. The mean value and the standard error are shown.

Table 1. Primers used for PCR and qPCR analysis

Target gene	Primer name	Sequence 5´→3[b]	Amplicon size (bp)
tanA$_{Sg}$	803	*GGTGAAAACCTGTATTTCCAGGGC*tctagtacctctagctccagctcgc[a]	1750
	804	*ATCGATAAGCTTAGTTAGCTAtta*tgagttgtcagctaagctttgatta	
tanB$_{Sg}$	774	*TAACTTTAAGAAGGAGATATACATatg*tcgattaatcaatggatttttg	1465
	775	*GCTATTAATGATGATGATGATGATGATG*aacaatggcatccacccattg	
tanA$_{Sg}$	1552	TGGCACTTGCCCTTGAAAA	58
	1553	CCCCAAACCGTTGCAAAA	
GALLO_1608	1554	GCGATTGGTGGTGTCTTACTTG	64
	1555	GACCTTGTCCACCCATGAGGTA	
tanB$_{Sg}$	1556	CTTGCAGTCTGACTTGGAGGAA	65
	1557	CTTTTGCCCCTAAACAAATACGA	
GALLO_1610	1558	AGCCAATCCAAATGCCAAATA	63
	1559	GAAAGAGTTGGGAGTGTGATGACTT	
SgdD	1560	ATTGCTGGCGCTTTTGAAA	65
	1561	TCAATTTTTAACCATGTGCCAATC	
SgdB	1562	CGCCTGTACCAGCCTTTTACA	76
	1563	TCTAGTAATTTAGCAGTATTGTGGTCGAT	
SgdC	1564	CCACCCACTTGACCCATCAG	60
	1565	CCTCGTACACGGATGTGTTCTG	
GALLO_1614	1566	GTCGTCCGTTGGAGGTTGAT	65
	1567	TGTTGTTTTCTTCTTACGACGGATT	
GALLO_1615	1568	GCCTTCATCGTGCCACTTACA	59
	1569	CCACCGCAGGCTTTGAAA	
ldhA	1570	ACGGTGCCGTAGGTTCATCT	58
	1571	TCTTGTGCGATTCCTTGGTTT	
ldhB	1572	CCGCACGTTTCCGTCAAG	61
	1573	GCGTGTACAGAACGTGCATCTAC	
gyrB	1574	GCCGCTGAAGCAGATAAGATTT	65
	1575	TCGCGACGCGGTTCTAC	
16S	1576	GGGTGATCGGCCACACTG	

[a] The nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the *tanA*$_{Sg}$ and *tanB*$_{Sg}$ gene sequences are written in lowercase letters

Figure 1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R2
R1
OH
OH
TanASg
TanBSg
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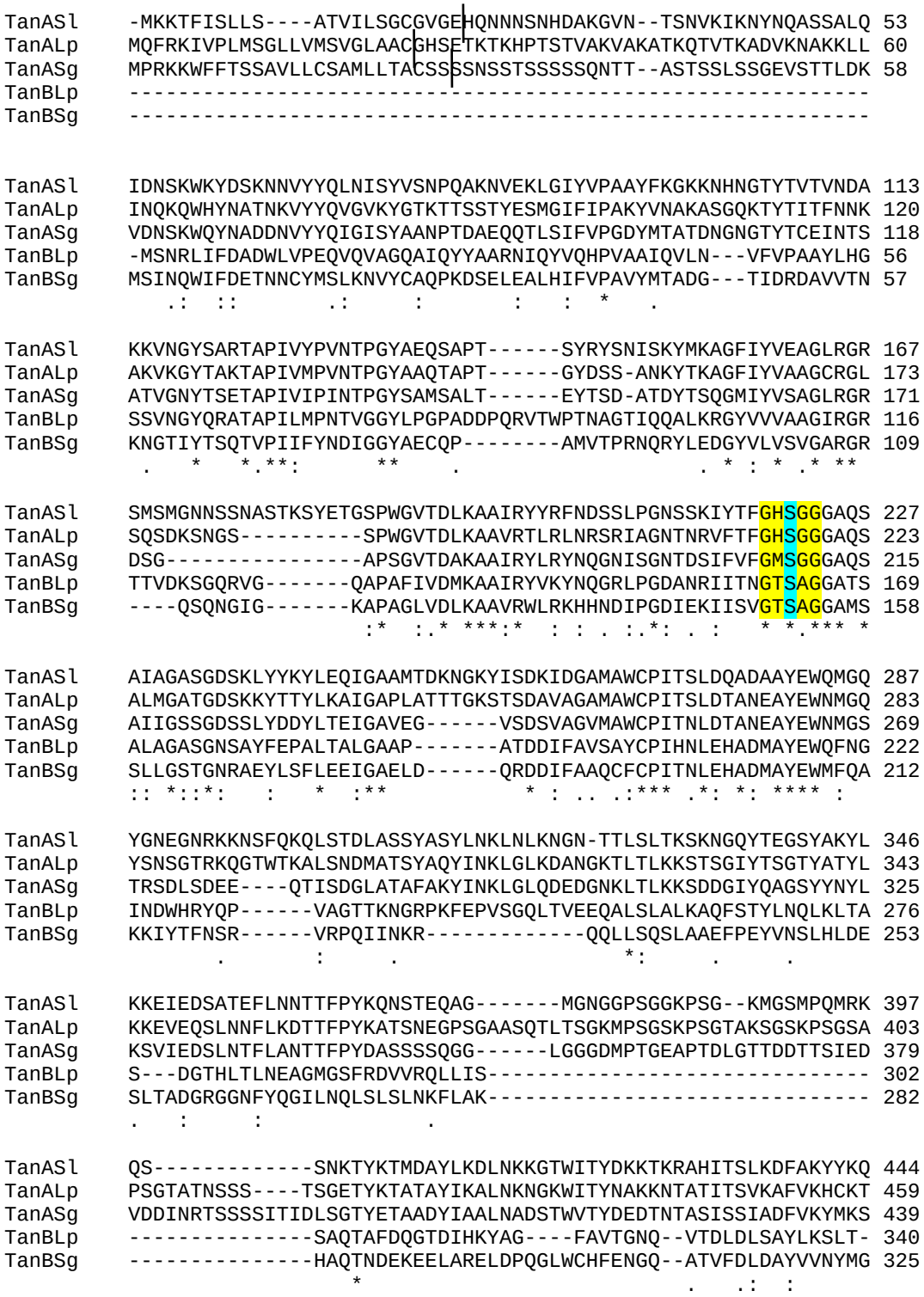
R1
OH
OH
SgdB
SgdC
SgdD
CO2
R1
OH
OH

Figure 2

A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TanBLp --
TanBSg --
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TanALp INQKQWHYNATNKVYYQVGVKYGTKTTSSTYESMGIFIPAKYVNAKASGQKTYTITFNNK 120
TanASg VDNSKWQYNADDNVYYQIGISYAANPTDAEQQTLSIFVPGDYMTATDNGNGTYTCEINTS 118
TanBLp -MSNRLIFDADWLVPEQVQVAGQAIQYYAARNIQYVQHPVAAIQVLN---VFVPAAYLHG 56
TanBSg MSINQWIFDETNNCYMSLKNVYCAQPKDSELEALHIFVPAVYMTADG---TIDRDAVVTN 57
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TanALp AKVKGYTAKTAPIVMPVNTPGYAAQTAPT------GYDSS-ANKYTKAGFIYVAAGCRGL 173
TanASg ATVGNYTSETAPIVIPINTPGYSAMSALT------EYTSD-ATDYTSQGMIYVSAGLRGR 171
TanBLp SSVNGYQRATAPILMPNTVGGYLPGPADDPQRVTWPTNAGTIQQALKRGYVVVAAGIRGR 116
TanBSg KNGTIYTSQTVPIIFYNDIGGYAECQP--------AMVTPRNQRYLEDGYVLVSVGARGR 109
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TanASg    FTTDLVKTDSLGNDLTTRINMYTPLYYLTDYYGGENSSNVASYWRIRTGLSQGDTALTTE 546
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TanASg    VNLALALEN-YGVKDLDFATVWGEQHTEAEISGDSTSNFIDWVNQSLADNS 596
TanBLp    IILAIMLEN--HGYGIDFALPWDIPHSGDYDLG----DLFSWIDGLCQ--- 469
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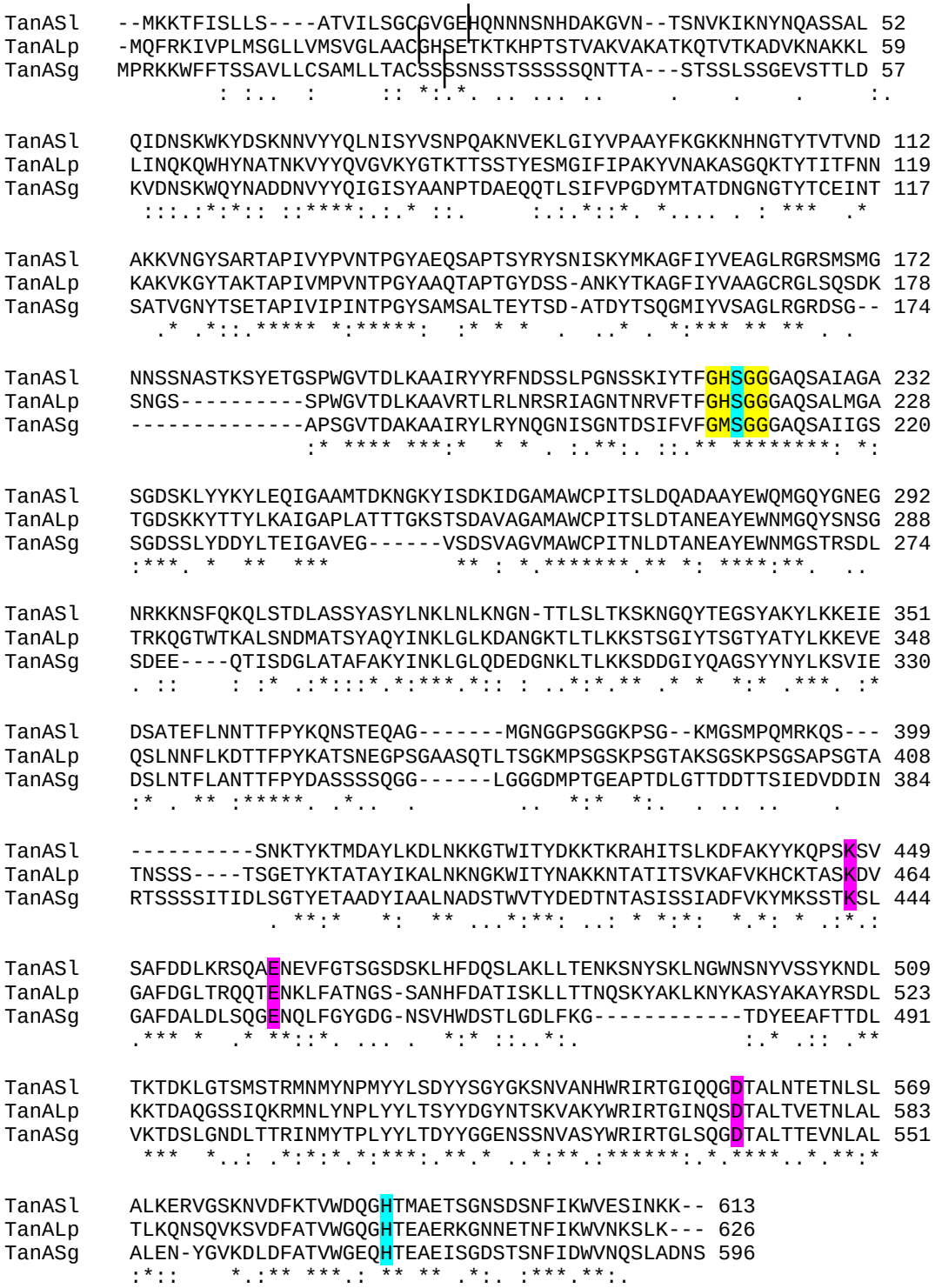
B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TanASg SGDSSLYDDYLTEIGAVEG------VSDSVAGVMAWCPITNLDTANEAYEWNMGSTRSDL 274
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C

```
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             *: ***       .:: .  *    :  :  ::  *.. : . ..:   * : ...

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          . *   *.**:: * :***    .        .  :   *: *: ***:*:.* ***

TanBLp    DKSGQRVGQAPAFIVDMKAAIRYVKYNQGRLPGDANRIITNGTSAGGATSALAGASGNSA 179
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          :* : :*:*** :**:***:*::: ::. :*** ::**: ******* *:* *::** *

TanBLp    YFEPALTALGAAP-ATDDIFAVSAYCPIHNLEHADMAYEWQFNGINDWHRYQPVAGTTKN 238
TanBSg    EYLSFLEEIGAELDQRDDIFAAQCFCPITNLEHADMAYEWMFQAKKIYTFNSRVRPQIIN 228
           : . *  :**     *****...:*** ********** *:. : :   . *     *

TanBLp    GRPKFEPVSGQLTVEEQALSLALKAQFSTYLNQLKLTAS---DGTHLTLNEAGMGSFRDV 295
TanBSg    KR------------QQLLSQSLAAEFPEYVNSLHLDESLTADGRGGNFYQGILNQLSLS 275
           *             :* ** :* *:*. *:*.*:*  *   **   .: :. :..:

TanBLp    VRQLLISSAQTAFDQGTDIHKYAG----FAVTGNQVTDLDLSAYLKSLT-RMKAVPAFDQ 350
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          :.::* . ***  ::    ::         . ..*.* :**.**: .   * *  ****.

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TanBSg    LDYQTPETEVFGNRDKNHRHFSENVAKHIEKLPALSDYQKAFQVDLAEEDLILARKLLNP 395
          **  :**.::**:   : :**:            *:: :.:. .:**: :** *   :**

TanBLp    LSYLTTTSS--QVAKHWRIRHGAADRDTSFAIPIILAIMLENHGYGIDFALPWDIPHSGD 454
TanBSg    MTFLQSDLEEKQVASHYRICLGAKDADTSFAISYLLALALKKRGIDVHYELIWGMGHADA 455
          :::* :  .  ***.*:**  ** * ******. :**: *:::* .:.: * *.: *:.

TanBLp    YDLGDLFSWIDGLCQ 469
TanBSg    DYNEEFSQWVDAIVH 470
              :: .*:*.: :
```

Figure 3

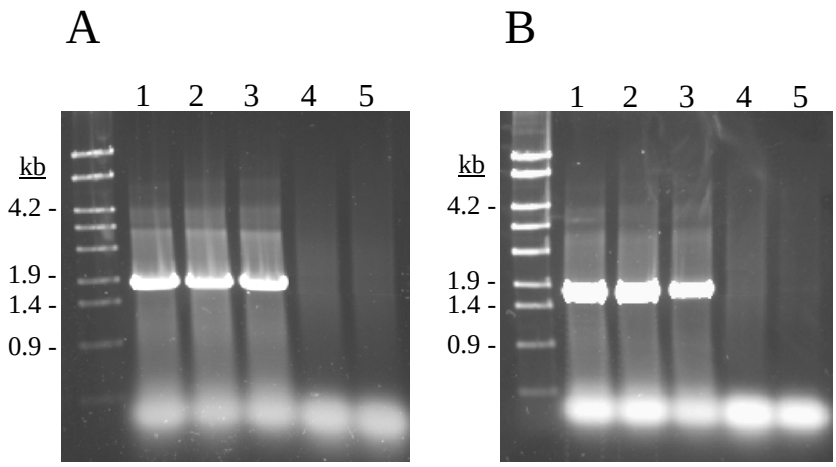
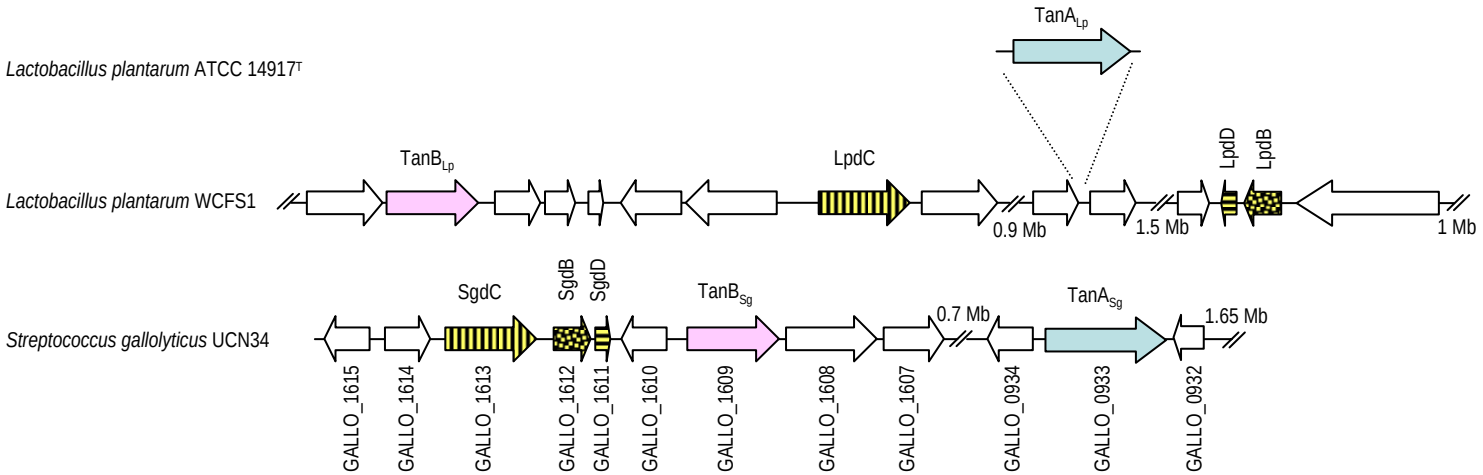


Figure 4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Lactobacillus plantarum ATCC 14917^{T}
TanB$_{Lp}$
LpdC
LpdD
LpdB
Lactobacillus plantarum WCFS1
0.9 Mb
1.5 Mb
1 Mb
SgdC
SgdB
SgdD
TanB$_{Sg}$
0.7 Mb
TanA$_{Sg}$
1.65 Mb
Streptococcus gallolyticus UCN34
GALLO_1615
GALLO_1614
GALLO_1613
GALLO_1612
GALLO_1611
GALLO_1610
GALLO_1609
GALLO_1608
GALLO_1607
GALLO_0934
GALLO_0933
GALLO_0932

Figure 5

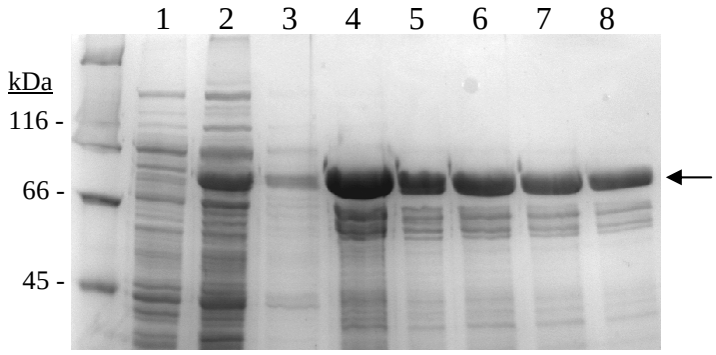


Figure 6

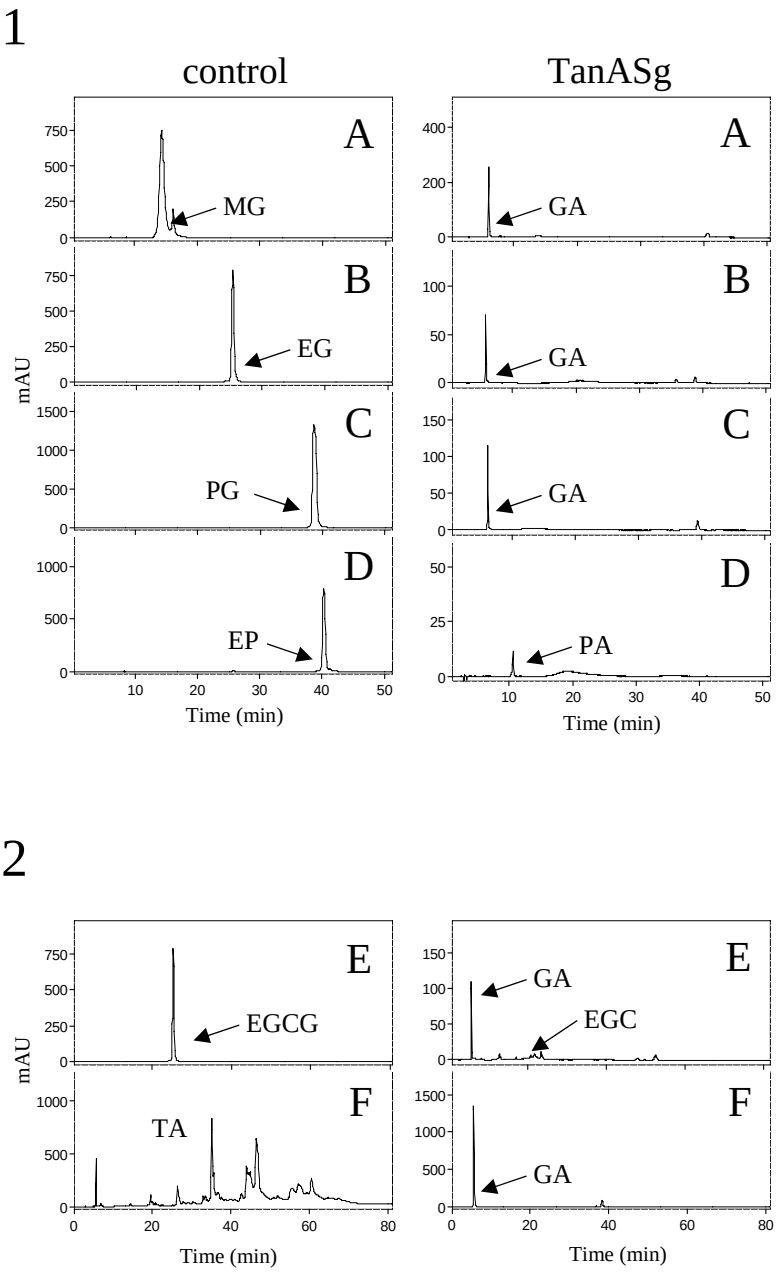
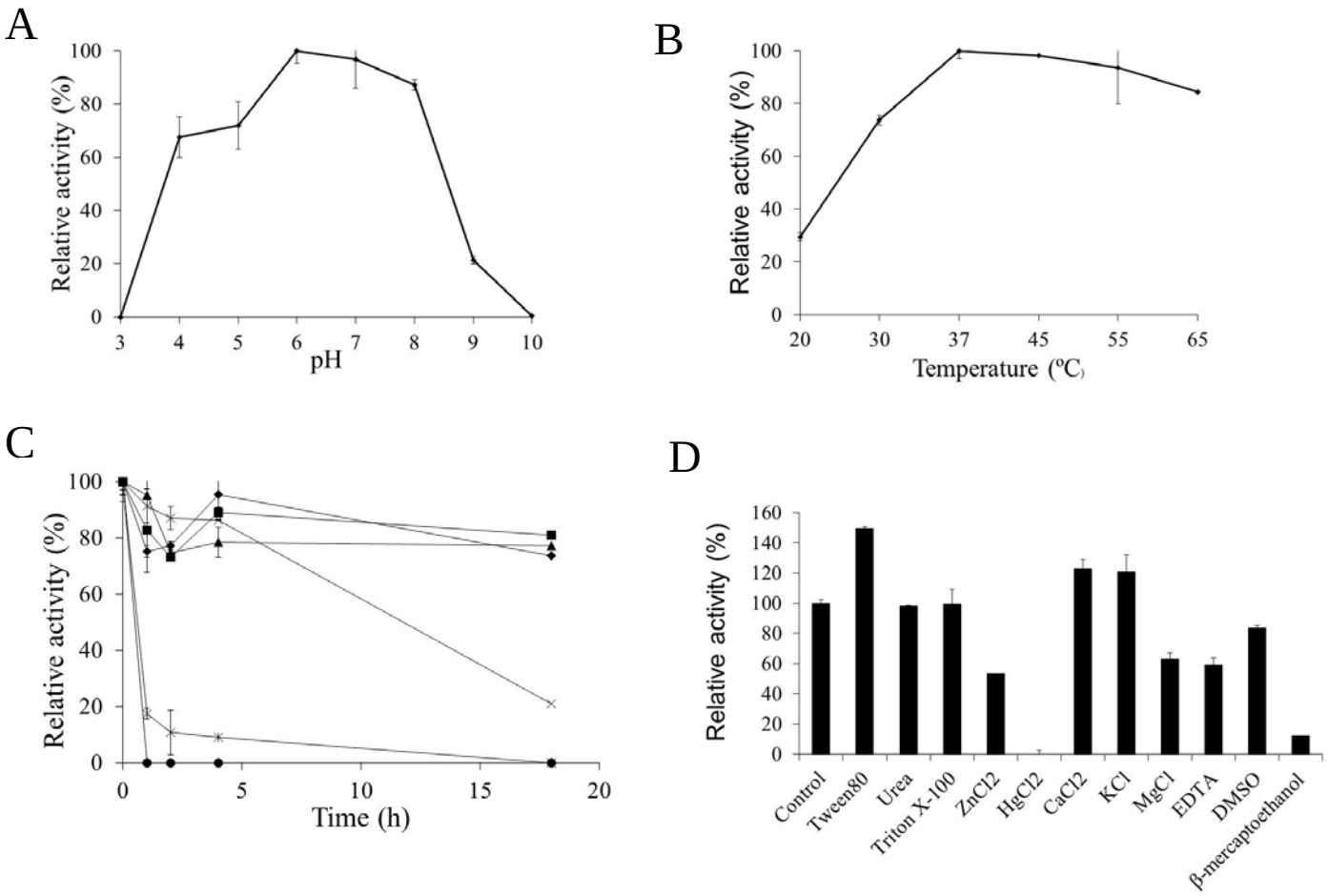

1
control
TanASg
A
B
C
D
MG
EG
PG
EP
GA
GA
GA
PA
mAU
Time (min)
Time (min)
2
E
F
EGCG
TA
GA
EGC
GA
mAU
Time (min)
Time (min)

Figure 7


A
Relative activity (%)
pH
B
Relative activity (%)
Temperature (ºC)
C
Relative activity (%)
Time (h)
D
Relative activity (%)
Control
Tween80
Urea
Triton X-100
ZnCl2
HgCl2
CaCl2
KCl
MgCl
EDTA
DMSO
β-mercaptoethanol

Resumen de los resultados

Es interesante conocer el efecto que ejerce la microbiota comensal en el intestino de mamíferos y el papel que la microbiota gastrointestinal desempeña en la salud y en las patologías humanas (Sekirov *et al*., 2010). Las bacterias se adaptan al estilo de vida y a la dieta del hospedador. Las actividades metabólicas específicas de las diferentes comunidades bacterianas, proporcionan al hospedador nuevos metabolitos que pueden aportar diferentes actividades funcionales. Por lo tanto tiene gran interés la identificación de las bacterias del TGI, sus capacidades metabólicas y los metabolitos que producen.

Por otro lado los compuestos fenólicos de la dieta son objeto de numerosos estudios debido a sus efectos beneficiosos frente a patologías asociadas con estrés oxidativo (Hooper *&* Frazier 2012). Dentro de los compuestos fenólicos, los taninos inhiben el crecimiento bacteriano modificando con ello la microbiota gastrointestinal. La capacidad que poseen ciertas bacterias para degradar taninos les permite ser más resistentes a estos compuestos y crecer mejor en su presencia. Estas bacterias degradadoras de taninos modifican el perfil de metabolitos los cuales son posteriormente absorbidos en el intestino del consumidor (Tuohy *et al*., 2012).

Aunque la capacidad para degradar galotaninos se ha descrito en algunas especies bacterianas, en la actualidad sólo se han identificado genéticamente las enzimas tanasa $TanA_{Sl}$ de *S. lugdunensis* (Noguchi *et al*., 2007) y $TanB_{Lp}$ de *L. plantarum* (Iwamoto *et al*., 2008). Con objeto de identificar bacterias que poseen proteínas con posible actividad tanasa, se realizó una búsqueda en las bases de datos de proteínas similares a $TanA_{Sl}$, la primera tanasa identificada genéticamente. Los resultados de la búsqueda indicaron que sólo un reducido número de proteínas poseían un grado de identidad superior al 25% (Tabla 2). Se ha considerado un 25% de identidad puesto que las dos únicas proteínas tanasas bacterianas identificadas en la actualidad, $TanA_{sg}$ y $TanB_{Lp}$, presentan un 27% de identidad, por lo que se puede suponer que proteínas que presentan un grado de identidad similar pueden poseer también actividad tanasa.

Tabla 2. Proteínas bacterianas similares a la tanasa $TanA_{Sl}$ de *S. lugdunensis*

Especie	Número de acceso	Número de aminoácidos	Identidad (%)
Staphylococcus lugdunensis	E6MB13 ($TanA_{Sl}$)	613	100
Oscillochloris trichoides	E1FN0	637	49
Dialister sp.	R7CT42	630	46
Atopobium parvulum	C8W7K9 ($TanA_{Ap}$)	607	40
Actinomyces johnsonii	U1S2R8	593	49
Methanolobus tindarius	W9DZK2	663	45
Clostridium butyricum	B1QUT6	633	48
Abiotrophia defectiva	W1Q1H2	585	46
Streptococcus gallolyticus	E0PJ49 ($TanA_{Sg}$)	596	43
Coriobacteriaceae bacterium	U2VB24	628	38
Olsenella sp.	G5FG76	651	38
Lactobacillus plantarum	D7VBF4 ($TanA_{Lp}$)	626	51
Lactobacillus pentosus	F6IRL4	627	51
Actinomyces sp.	E7NAA2	609	47
Streptococcus gallolyticus	F0VX82 ($TanB_{Sg}$)	349	46
Fusobacterium nerophorum	H1D7U2	423	27
Streptomyces avermitilis	Q82MV9	566	27
Clostridium butyricum	C41BL3	528	28
Oenococcus oeni	J3F602	425	28
Lactobacillus pentosus	T2HN93	470	29
Lactobacillus plantarum	B9A0W2 ($TanB_{Lp}$)	469	27
Lactobacillus paraplantarum	T24NW8	469	25
Aggregatibacter aphrophilus	G4BCT8	513	26
Fusobacterium nucleatum	U7THI3	521	27

Tal como muestra la Tabla 2, la mayoría de las proteínas similares a $TanA_{Sl}$ pertenecen a bacterias no presentes en el TGI humano o a bacterias no cultivables. Puesto que la composición de las comunidades bacterianas varía a lo largo del TGI humano (Sekirov *et al*., 2010) y con objeto de conocer el posible papel de las proteínas similares a $TanA_{Sl}$ en la degradación de los galotaninos presentes en la dieta, se decidió estudiar las proteínas pertenecientes a las especies *A. parvulum*, *L. plantarum* y *S. gallolyticus* por ser especies presentes en el TGI humano. La especie *A. parvulum* es una bacteria aislada de la cavidad oral (Copeland *et al*., 2009; Riggio *et al*., 2008), las cepas de *L. plantarum* se pueden localizar en la cavidad oral o en el intestino delgado (ileón), y las bacterias de la especie *S. gallolyticus* se han aislado en el intestino grueso humano (colon y recto).

Al realizar un alineamiento de la proteína $TanA_{Sl}$ de *S. lugdunenis* con las proteínas de *A. parvulum*, *L. plantarum* y *S. gallolyticus* (Figura 11) se comprobó que existían dos grupos de proteínas bien diferenciados. Por un lado, un grupo de proteínas de una longitud de 600

aminoácidos, aproximadamente, en el que se encuentran $TanA_{Sl}$, la proteína de *A. parvulum* (acceso C8W7K9), una proteína de *L. plantarum* (acceso D7VVB4) y otra proteína de *S. gallolyticus* (acceso E0PJH2). Por otro lado, en otro grupo, se encuentran dos proteínas de menos de 500 aminoácidos, la enzima tanasa de *L. plantarum* previamente descrita (acceso B9A0W2, TanLp1 o $TanB_{Lp}$) y una proteína de *S. gallolyticus* (acceso F0VX82). Puesto que cuando se identificó la proteína de *S. lugdunensis* se le denominó "TanA" se decidió nombrar a todas las proteínas de este grupo como "TanA" seguido de las iniciales de la especie a la que pertenecen. Por ello, las proteínas incluidas en el segundo grupo, se les denominó "TanB", a pesar de que la proteína de *L. plantarum* se denominó inicialmente como TanLp1. Las proteínas TanA presentan entre ellas un grado de identidad entre 39 y 50%, mientras que la identidad entre las proteínas TanB es de un 32%. El grado de identidad entre proteínas TanA y TanB es igual o inferior al 30% (Tabla 3).

A

```
TanASl   -MKKTFISLLS----ATVILSGCGVGEHQNNNSNHDAKGVN--TSNVKIKNYNQASSALQ 53
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TanASg   MPRKKWFFTSSAVLLCSAMLLTACSSSSNSSTSSSSSQNTT--ASTSSLSSGEVSTTLDK 58
TanAAp   MAFTRKEFLSLSALGVAGTLAACTPQARTSDNTNQPASNVD-----------LEEFKSLK 49
TanBLp   ------------------------------------------------------------
TanBSg   ------------------------------------------------------------

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TanALp   INQKQWHYNATNKVYYQVGVKYGTKTTSSTYESMGIFIPAKYVNAKASGQKTYTITFNNK 120
TanASg   VDNSKWQYNADDNVYYQIGISYAANPTDAEQQTLSIFVPGDYMTATDNGNGTYTCEINTS 118
TanAAp   LDMTQWSYDEDNDCYYQLGIQYCTKPASKSVNTLSVFVPGKYFSGKKNGS-TYECEVSEK 108
TanBLp   -MSNRLIFDADWLVPEQVQVAGQAIQYYAARNIQYVQHPVAAIQVLNVFVPAAYLHG--- 56
TanBSg   MSINQWIFDETNNCYMSLKNVYCAQPKDSELEALHIFVPAVYMTADGTIDRDAVVTN--- 57
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TanASl   KKVNGYSARTAPIVYPVNTPGYAEQSAPT------SYRYSNISKYMKAGFIYVEAGLRGR 167
TanALp   AKVKGYTAKTAPIVMPVNTPGYAAQTAPT------GYDSS-ANKYTKAGFIYVAAGCRGL 173
TanASg   ATVGNYTSETAPIVIPINTPGYSAMSALT------EYTSD-ATDYTSQGMIYVSAGLRGR 171
TanAAp   AVVGSFTARTAPIVMPINTATLFPQSAPT------SYAYEGLAPYLEAGFVYVYAGFRGR 162
TanBLp   SSVNGYQRATAPILMPNTVGGYLPGPADDPQRVTWPTNAGTIQQALKRGYVVVAAGIRGR 116
TanBSg   KNGTIYTSQTVPIIFYNDIGGYAECQP--------AMVTPRNQRYLEDGYVLVSVGARGR 109
              :   *.**:           .                  . * : * .* **

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TanASg   DSG----------------APSGVTDAKAAIRYLRYNQGNISGNTDSIFVFGMSGGGAQS 215
TanAAp   SAGYDSTTG--SDELYAGGSPWPAVDFKAAIRYLRYNNELLPCNTSKIFVFGFAAGGGLS 220
TanBLp   TTVDKSGQRVG-------QAPAFIVDMKAAIRYVKYNQGRLPGDANRIITNGTSAGGATS 169
TanBSg   ----QSQNGIG-------KAPAGLVDLKAAVRWLRKHHNDIPGDIEKIISVGTSAGGAMS 158
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TanASg   AIIGSSGDSSLYDDYLTEIGAVEG------VSDSVAGVMAWCPITNLDTANEAYEWNMGS 269
TanAAp   AVLGTSGDSSLYMPYLKAVGAATHSEKGEQLSDSIYGSASWCPATSYDLADAAYEWSAGQ 280
TanBLp   ALAGASGNSAYFEPALTALGAAP-------ATDDIFAVSAYCPIHNLEHADMAYEWQFNG 222
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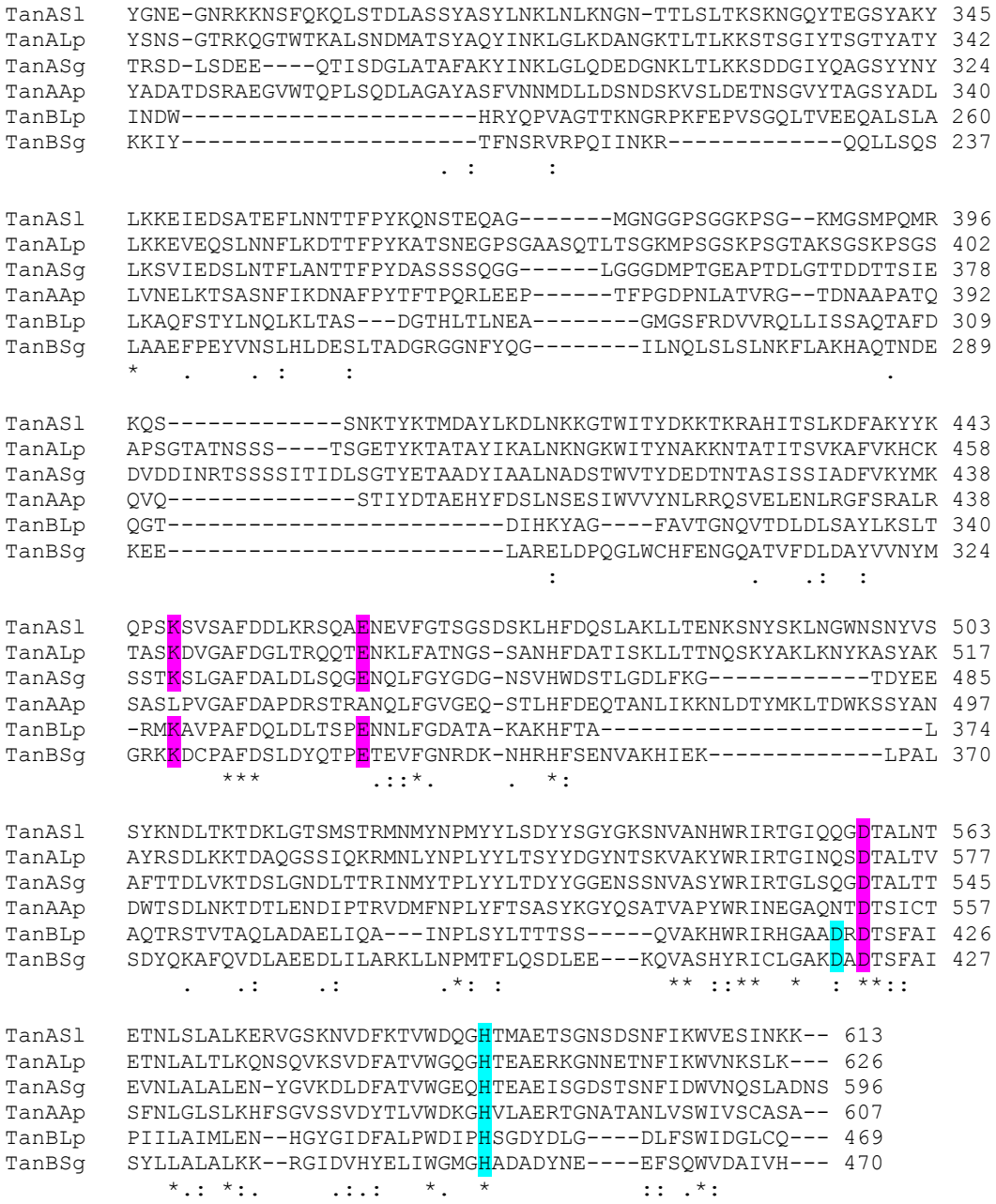
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TanAAp YADATDSRAEGVWTQPLSQDLAGAYASFVNNMDLLDSNDSKVSLDETNSGVYTAGSYADL 340
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TanALp LKKEVEQSLNNFLKDTTFPYKATSNEGPSGAASQTLTSGKMPSGSKPSGTAKSGSKPSGS 402
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TanBSg LAAEFPEYVNSLHLDESLTADGRGGNFYQG--------ILNQLSLSLNKFLAKHAQTNDE 289
TanASl KQS----------SNKTYKTMDAYLKDLNKKGTWITYDKKTKRAHITSLKDFAKYYK 443
TanALp APSGTATNSSS----TSGETYKTATAYIKALNKNGKWITYNAKKNTATITSVKAFVKHCK 458
TanASg DVDDINRTSSSSITIDLSGTYETAADYIAALNADSTWVTYDEDTNTASISSIADFVKYMK 438
TanAAp QVQ----------STIYDTAEHYFDSLNSESIWVVYNLRRQSVELENLRGFSRALR 438
TanBLp QGT----------DIHKYAG----FAVTGNQVTDLDLSAYLKSLT 340
TanBSg KEE----------LARELDPQGLWCHFENGQATVFDLDAYVVNYM 324
TanASl QPSKSVSAFDDLKRSQAENEVFGTSGSDSKLHFDQSLAKLLTENKSNYSKLNGWNSNYVS 503
TanALp TASKDVGAFDGLTRQQTENKLFATNGS-SANHFDATISKLLTTNQSKYAKLKNYKASYAK 517
TanASg SSTKSLGAFDALDLSQGENQLFGYGDG-NSVHWDSTLGDLFKG----------TDYEE 485
TanAAp SASLPVGAFDAPDRSTRANQLFGVGEQ-STLHFDEQTANLIKKNLDTYMKLTDWKSSYAN 497
TanBLp -RMKAVPAFDQLDLTSPENNLFGDATA-KAKHFTA----------L 374
TanBSg GRKKDCPAFDSLDYQTPETEVFGNRDK-NHRHFSENVAKHIEK----------LPAL 370
TanASl SYKNDLTKTDKLGTSMSTRMNMYNPMYYLSDYYSGYGKSNVANHWRIRTGIQQGDTALNT 563
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TanASg AFTTDLVKTDSLGNDLTTRINMYTPLYYLTDYYGGENSSNVASYWRIRTGLSQGDTALTT 545
TanAAp DWTSDLNKTDTLENDIPTRVDMFNPLYFTSASYKGYQSATVAPYWRINEGAQNTDTSICT 557
TanBLp AQTRSTVTAQLADAELIQA---INPLSYLTTTSS-----QVAKHWRIRHGAADRDTSFAI 426
TanBSg SDYQKAFQVDLAEEDLILARKLLNPMTFLQSDLEE---KQVASHYRICLGAKDADTSFAI 427
TanASl ETNLSLALKERVGSKNVDFKTVWDQGHTMAETSGNSDSNFIKWVESINKK-- 613
TanALp ETNLALTLKQNSQVKSVDFATVWGQGHTEAERKGNNETNFIKWVNKSLK--- 626
TanASg EVNLALALEN-YGVKDLDFATVWGEQHTEAEISGDSTSNFIDWVNQSLADNS 596
TanAAp SFNLGLSLKHFSGVSSVDYTLVWDKGHVLAERTGNATANLVSWIVSCASA-- 607
TanBLp PIILAIMLEN--HGYGIDFALPWDIPHSGDYDLG----DLFSWIDGLCQ--- 469
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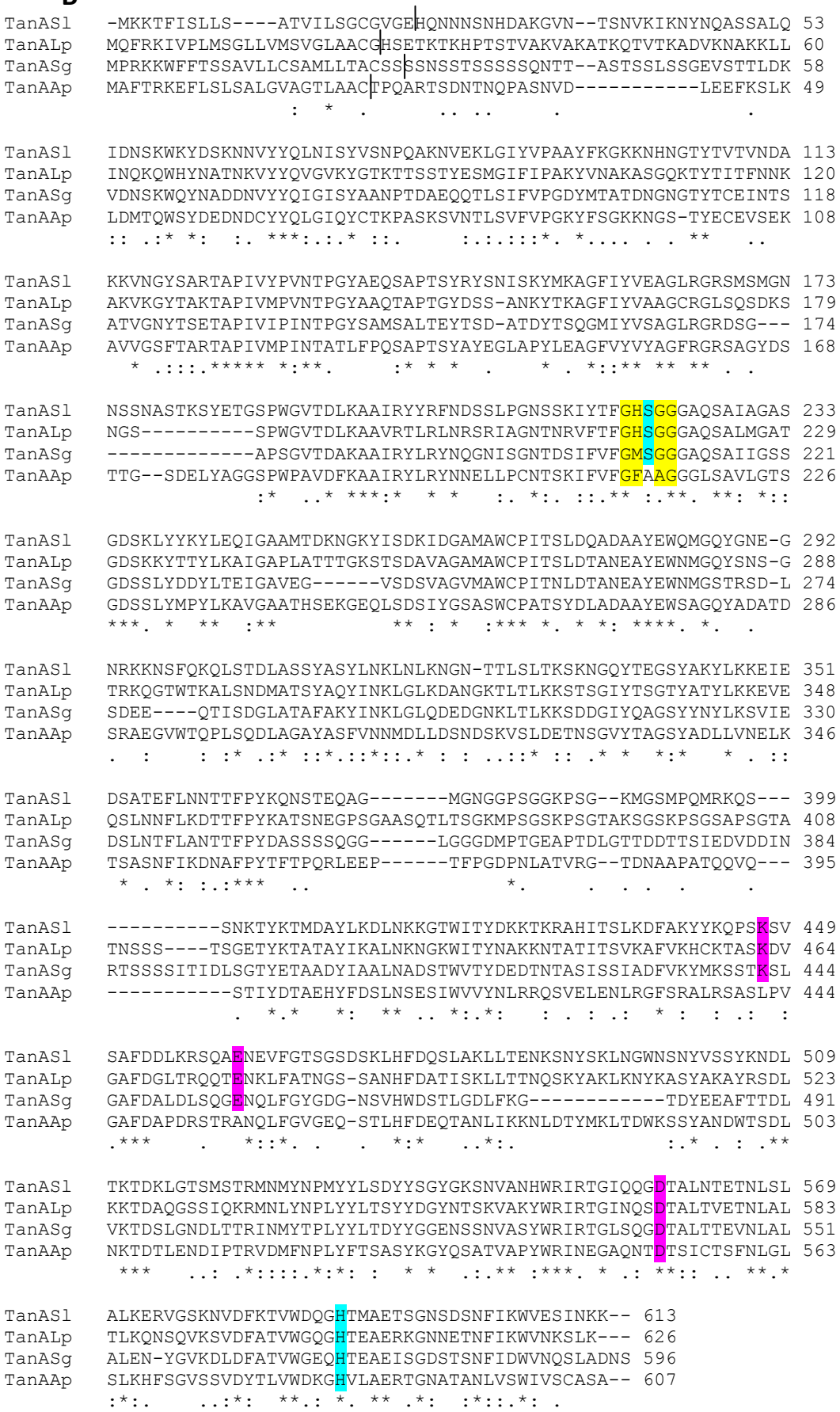
B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TanALp TNSSS----TSGETYKTATAYIKALNKNGKWITYNAKKNTATITSVKAFVKHCKTASKDV 464
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TanAAp -----------STIYDTAEHYFDSLNSESIWVVYNLRRQSVELENLRGFSRALRSASLPV 444
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TanALp GAFDGLTRQQTENKLFATNGS-SANHFDATISKLLTTNQSKYAKLKNYKASYAKAYRSDL 523
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TanASg ALEN-YGVKDLDFATVWGEQHTEAEISGDSTSNFIDWVNQSLADNS 596
TanAAp SLKHFSGVSSVDYTLVWDKGHVLAERTGNATANLVSWIVSCASA-- 607

C

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TanBLp   -MSNRLIFDADWLVPEQVQVAGQAIQYYAARNIQYVQHPVAAIQVLNVFVPAAYLHGSSV 59
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TanBSg   TIYTSQTVPIIFYNDIGGYAECQP--------AMVTPRNQRYLEDGYVLVSVGARGR--- 109
TanBLp   NGYQRATAPILMPNTVGGYLPGPADDPQRVTWPTNAGTIQQALKRGYVVVAAGIRGRTTV 119
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TanBSg   -QSQNGIGKAPAGLVDLKAAVRWLRKHHNDIPGDIEKIISVGTSAGGAMSSLLGSTGNRA 168
TanBLp   DKSGQRVGQAPAFIVDMKAAIRYVKYNQGRLPGDANRIITNGTSAGGATSALAGASGNSA 179
          :* : :*:*** :**:***:*::: ::. :*** ::**: ******* *:* *::** *

TanBSg   EYLSFLEEIGAELDQRDDIFAAQCFCPITNLEHADMAYEWMFQAKKIYTFNSRVRPQIIN 228
TanBLp   YFEPALTALGAAP-ATDDIFAVSAYCPIHNLEHADMAYEWQFNGINDWHRYQPVAGTTKN 238
          : . *  :**     *****...:*** ********** *:. : :   . *     *

TanBSg   KR-------------QQLLSQSLAAEFPEYVNSLHLDESLTADGRGGNFYQGILNQLSLS 275
TanBLp   GRPKFEPVSGQLTVEEQALSLALKAQFSTYLNQLKLTAS---DGTHLTLNEAGMGSFRDV 295
          *             :* ** :* *:*. *:*.*:*  *   **   .: :. :..:

TanBSg   LNKFLAKHAQTNDEKEELARELDPQGLWCHFENGQATVFDLDAYVVNYMGRKKDCPAFDS 335
TanBLp   VRQLLISSAQTAFDQGTDIHKYAG----FAVTGNQVTDLDLSAYLKSLT-RMKAVPAFDQ 350
         :.::* . ***  ::    ::         . ..*.* :**.**: .   * *  ****.

TanBSg   LDYQTPETEVFGNRDKNHRHFSENVAKHIEKLPALSDYQKAFQVDLAEEDLILARKLLNP 395
TanBLp   LDLTSPENNLFGDATAKAKHFTA----------LAQTRSTVTAQLADAELIQA---INP 396
         **  :**.::**:   : :**:            *:: :.:. .:**: :** *   :**

TanBSg   MTFLQSDLEEKQVASHYRICLGAKDADTSFAISYLLALALKKRGIDVHYELIWGMGHADA 455
TanBLp   LSYLTTTSS--QVAKHWRIRHGAADRDTSFAIPIILAIMLENHGYGIDFALPWDIPHSGD 454
         :::* :  .  ***.*:**  ** * ******. :**: *:::* .:.: * *.: *:.

TanBSg   DYNEEFSQWVDAIVH 470
TanBLp   YDLGDLFSWIDGLCQ 469
             :: .*:*.: :
```

Figura 11. Comparación de las secuencias de aa de las proteínas tanasas de *S. lugdunensis* ($TanA_{Sl}$), *A. parvulum* ($TanA_{Ap}$), *L. plantarum (*$TanA_{Lp}$ y $TanB_{Lp}$*)* y *S. gallolyticus (*$TanA_{Sg}$ y $TanB_{Sg}$*).* Los residuos idénticos se indican con (*), los residuos conservados con (:) y los semiconservados con (.). Los motivos conservados de la serin-hidrolasas se encuentran marcados en color amarillo; Los residuos de la triada catalítica que son idénticos a los de TanBLp se encuentran marcados en azul y los residuos que intervienen en la unión al substrato se encuentran marcados en color rosa.

Tabla 3. Grado de identidad entre proteínas TanA y TanB.

$TanA_{Sl}$	$TanA_{Lp}$	$TanA_{Sg}$	$TanA_{Ap}$	$TanB_{Sg}$	$TanB_{Lp}$		
E6MB13	D7VVB4	E0PJH2	C8W7K9	F0VX82	B9A0W2	Protein	
100	50	49	39	29	27	E6MB13	$TanA_{Sl}$
	100	46	36	29	27	D7VBF4	$TanA_{Lp}$
		100	36	29	30	E0PJH2	$TanA_{Sg}$
			100	27	26	C8W7K9	$TanA_{Ap}$
				100	32	F0VX82	$TanB_{Sg}$
					100	B9A0W2	$TanB_{Lp}$

Para conocer el metabolismo de galotaninos en las especies presentes en el TGI humano que poseen enzimas TanA o TanB (*A. parvumum* en cavidad oral, *L. plantarum* en cavidad oral e intestino delgado y *S. gallolyticus* en intestino grueso) se decidió clonar los genes que las codifican y caracterizar bioquímicamente las proteínas tanasas producidas.

1. *Atopobium parvulum*

A. parvulum se describió por primera vez por Weiberg *et al*. (1937) como *Streptoccoccus parvulum* y en 1.992 se reclasificó como *Atopobium parvulum*. *A. parvulum* es una bacteria Gram+, que pertenece a la rama *Atopobium*/*Olsenella* de la familia *Coriobacteriaceae* (Copeland *et al*., 2009). Se trata de una especie bacteriana aislada frecuentemente de la cavidad oral humana, especialmente del dorso de la lengua y asociada con halitosis. Puesto que *A. parvulum* se encuentra en la cavidad oral y posee una proteína 40% idéntica a $TanA_{Sl}$ es interesante conocer si durante el paso de los alimentos por la cavidad oral, esta proteína participa en la degradación de los galotaninos presentes en la dieta.

La cepa *A. parvulum* DSM 20469^T cuyo genoma se ha secuenciado completamente, posee el gen *apar_1020* que codifica una proteína ($TanA_{Ap}$) anotada como esterasa/lipasa. $TanA_{Ap}$ posee un 39% de identidad con $TanA_{Sl}$ y un 26% de identidad con $TanB_{Lp}$ (Figura 11, tabla 3). El estudio de su secuencia de aminoácidos predijo la existencia de un péptido señal de 23 aminoácidos por lo que la proteína $TanA_{Ap}$ parece ser una proteína extracelular. La proteína $TanA_{Ap}$ madura posee 584 aminoácidos, un peso molecular de 63,8 kDa y un punto isoeléctrico de 4,62.

El gen *apar_1020* que codifica la tanasa $TanA_{Ap}$ se clonó en un vector de expresión para hiperproducir la proteína $TanA_{Ap}$ recombinante pura (Figura 1, capítulo 1). La proteína $TanA_{Ap}$ presentó una baja actividad específica frente a galato de metilo. Su actividad específica fue de 3,5 U/mg, 116 veces menor que la actividad específica que presentó $TanB_{Lp}$. La actividad bioquímica de $TanA_{Ap}$ se caracterizó mediante la utilización de un método colorimétrico con rodanina. Utilizando este método la tanasa $TanA_{Ap}$ de *A. parvulum* presentó un máximo de actividad a pH 6, manteniendo actividades superiores al 70% a pH 7 y 8 (Figura 2 A, capítulo 1). En el intervalo de temperaturas estudiado, $TanA_{Ap}$ presenta una actividad óptima a 50 ºC, aunque a 37 ºC, la temperatura fisiológica en humanos, presentó el 80% de su actividad máxima. También presentó el 80% de actividad a 20, 42 y 65 ºC (Figura 2 B, capítulo 1). La

proteína mantuvo más del 60% de su actividad máxima después de una incubación a 45 ºC durante 18 h (Figura 2 C, capítulo 1).

Para conocer el rango de substratos de la enzima, ésta se incubó en presencia de posibles substratos, y los productos de la reacción se analizaron mediante HPLC. $TanA_{Ap}$ no fue capaz de hidrolizar en su totalidad ninguno de los ésteres ensayados. De los substratos analizados tan sólo hidrolizó mínimamente galato de metilo, galato de etilo, galato de propilo y protocatecuato de etilo (Figura 3, capítulo 1). Aunque la enzima presentó una especificidad de substrato similar a la que presentó $TanB_{Lp}$ (hidrólisis de ésteres de los ácidos gálico y protocatéquico), la baja actividad específica que presenta $TanA_{Ap}$ ocasionó sólo una mínima hidrólisis de los ésteres ensayados.

Puesto que en *L. plantarum* se ha descrito que la proteína Lp_2945 (LpdC) está implicada en la descarboxilación del ácido gálico a pirogalol, segunda etapa en la degradación de galotaninos, se realizó una búsqueda de proteínas similares en la cepa *A. parvulum* DSM 20469^T cuyo genoma se ha secuenciado completamente. La búsqueda reveló que en *A. parvulum* no existen proteínas similares a la posible galato descarboxilasa de *L. plantarum*.

2. *Lactobacillus plantarum*

L. plantarum es una bacteria Gram+ y catalasa negativa que pertenece al grupo de bacterias lácticas. *L. plantarum* además de ser un microorganismo presente en el TGI, también se adquiere por la ingesta de alimentos vegetales fermentados, donde los taninos son abundantes (Siezen *et al*., 2010). Algunas cepas se han seleccionado como probióticos debido a su resistencia a atravesar el TGI superior (Siezen *et al*., 2010). Se ha descrito que las bacterias del género *Lactobacillus* se encuentran principalmente en estómago e intestino delgado, sin embargo también se han encontrado, en niveles inferiores, en el colon y en las heces (Kleerebezem & Vaughan, 2009). *L. plantarum* además de presentar una gran adaptabilidad a nichos en los que los taninos son abundantes, también está presente a lo largo del TGI, convirtiéndose en una bacteria con gran potencial para interaccionar con los compuestos fenólicos de la dieta y modular sus efectos en el consumidor.

La tanasa $TanB_{Lp}$ de *L. plantarum* se identificó debido al 27% de identidad que presenta con la enzima $TanA_{Sl}$ previamente descrita (Iwamoto *et al*., 2008). Al realizar el alineamiento de las proteínas similares a $TanA_{Sl}$ (Tabla 2) se observó la existencia de una segunda proteína

de *L. plantarum* que presentó un 50% de identidad con $TanA_{Sl}$. Además ambas proteínas compartían otras características puesto que ambas proteínas presentan un tamaño de 67 kDa, poseen puntos isoeléctricos alcalinos (9,54 para $TanA_{Sl}$ y 9,94 para $TanA_{Lp}$) y son extracelulares ya que presentan una secuencia que codifica un péptido señal.

En 36 cepas de la especie *L. plantarum* se estudió, mediante PCR, la presencia de los genes que codifican las dos enzimas tanasa (*tanA*$_{Lp}$ y *tanB*$_{Lp}$). El estudio reveló que todas las cepas de *L. plantarum* analizadas presentaron el gen que codifica $TanB_{Lp}$, pero sólo 4 cepas de *L. plantarum* (entre ellas la cepa tipo ATCC 14917^T o CECT 748^T) amplificaron el gen *tanA*$_{Lp}$. Al contrario que la proteína $TanB_{Lp}$ que no posee péptido señal, la proteína $TanA_{Lp}$ es una proteína extracelular ya que posee un posible péptido señal. La actividad tanasa extracelular debida a la presencia de $TanA_{Lp}$ se comprobó al incubar cepas de *L. plantarum* que poseen sólo $TanB_{Lp}$ (como la cepa *L. plantarum* WCFS1) y cepas que poseen las dos tanasas (como la cepa *L. plantarum* ATCC 14917^T) en presencia de un galotanino de alto peso molecular que no puede pasar al interior celular. Se verificó que sólo la cepa que posee $TanA_{Lp}$ funcional fue capaz de degradar el galotanino ensayado (Figura 2, capítulo 2).

Con objeto de conocer la función de ambas tanasas en el metabolismo de galotaninos de *L. plantarum* se estudió la expresión de ambos genes en respuesta a la presencia de un galotanino sencillo, como galato de metilo. Se comprobó que ambos genes presentaron un comportamiento diferente, la expresión del gen *tanB*$_{Lp}$ se indujo en presencia de galato de metilo, mientras que la expresión de gen *tanA*$_{Lp}$ no se modificó.

Las dos enzimas tanasas presentes en algunas cepas de *L .plantarum* presentaron diferente respuesta a la presencia de un éster del ácido gálico, sin embargo no se conoce si sus propiedades bioquímicas son similares o diferentes. Puesto que la caracterización bioquímica de la proteína $TanB_{Lp}$ se llevó a cabo antes de la realización de esta tesis, en este trabajo se decidió caracterizar la proteína $TanA_{Lp}$. El gen *tanA*$_{Lp}$ de *L. plantarum* ATCC 14917^T que codifica la tanasa $TanA_{Lp}$ se clonó en un vector de expresión con objeto de hiperproducir la proteína $TanA_{Lp}$ recombinante pura. La proteína $TanA_{Lp}$ presentó una actividad específica de 39 U/mg frente a galato de metilo. La actividad óptima de la proteína $TanA_{Lp}$ fue a 20-30 ºC y a pH 6.0 (Figura 4 A y B, capítulo 2). La presencia de Ca^{2+} produjo un aumento en la actividad de $TanA_{Lp}$, mientras que la presencia de Hg^{2+} inhibió completamente su actividad (Figura 4 D, capítulo 2). Respecto a la especificidad de substrato $TanA_{Lp}$ sólo hidrolizó ésteres de los ácidos gálico y protocatéquico que poseen el alcohol susbtituyente con una cadena alifática corta.

L. plantarum degrada galotaninos mediante la acción secuencial de la enzima tanasa ($TanA_{Lp}$ o $TanB_{Lp}$) y la enzima galato descarboxilasa o descarboxilasa de ácido gálico (Osawa *et al*., 2000; Rodríguez *et al*., 2008 a). Una vez caracterizadas en *L. plantarum* las enzimas que participan en la primera etapa de la degradación de galotaninos se abordó el estudio genético y bioquímico de la enzima responsable de la descarboxilación de ácido gálico para originar pirogalol. La enzima galato descarboxilasa forma parte de las descarboxilasas no-oxidativas de ácidos aromáticos. Estas descarboxilasas presentan una organización genética común caracterizada por la presencia de tres genes agrupados en un mismo operón. En el inicio de esta tesis Curiel (2010) describió que la proteína Lp_2945, incorrectamente anotada como 3-octaprenil-4-hidroxibenzoato descarboxilasa o UbiD en el genoma de *L. plantarum* WCFS1, estaba implicada en la actividad galato descarboxilasa, puesto que se indujo en presencia de ácido gálico y su interrupción originó la desaparición de la actividad enzimática (Curiel, 2010). La proteína Lp_2945 presentó alta identidad con las subunidades C de las descarboxilasas no-oxidativas, por lo que se la denominó LpdC (*Lactobacillus plantarum* descarboxilasa, subunidad C). La búsqueda en el genoma de *L. plantarum* WCFS1 de proteínas similares a las que codifican las subunidades B y D de las descarboxilasas no-oxidativas reveló que las subunidades B y D estaban codificadas por los genes *lp_0271* (*lpdB*) y *lp*_0272 (*lpdD*), respectivamente. *L. plantarum* es la única bacteria en la que los tres genes que codifican la descarboxilasa se encuentran separados 1 Mb en el cromosoma por lo que no forman parte del mismo operon (Figura 2, capítulo 3). Señalar que el gen que codifica la subunidad C (LpdC) y que se indujo en presencia de ácido gálico, *lpdC* o *lp_2945,* se localiza próximo al gen que codifica la enzima $TanB_{Lp}$ (*lp_2956*), también inducida por un éster del ácido gálico (Figura 2, capítulo 3).

Para conocer el papel que desempeña cada una de las subunidades en la descarboxilación del ácido gálico se llevaron a cabo varias aproximaciones experimentales. La combinación de extractos de *E. coli* en los que se hiperprodujo cada una de las subunidades de la enzima galato descarboxilasa demostraron que la subunidad C (LpdC) es la única subunidad que se requirió para obtener actividad galato descarboxilasa (Figura 3 C, capítulo 3). En estos experimentos en los que se utilizó extractos de *E. coli,* no se puede descartar la presencia de proteínas de *E. coli* que puedan suplir la función de alguna de las subunidades. Estos resultados contrastan con los obtenidos cuando se interrumpió en *L. plantarum* cada uno de los genes que codifican las subunidades de la descarboxilasa. Los mutantes de *L. plantarum* obtenidos revelaron que para obtener actividad enzimática era necesario que los genes *lpdB* y

lpdC fueran funcionales (Figura 5, capítulo 3). Los estudios con estos mutantes revelaron además que el gen *lpdD* no era necesario para la actividad galato descarboxilasa en *L. plantarum*, sin embargo sí fue necesario que las proteínas LpdB y LpdC se sintetizasen en la misma cepa (Figura 5, capítulo 3). Los resultados obtenidos indicaron que LpdC es la única subunidad catalítica y que la subunidad LpdB es necesaria para la actividad, pudiendo tener un papel en la maduración o plegamiento de LpdC.

3. *Streptococcus gallolyticus*

S. gallolyticus es una bacteria Gram+ y catalasa negativa que al igual que *L. plantarum* pertenece al grupo de bacterias lácticas. *S. gallolyticus* se ha aislado frecuentemente de la microbiota intestinal de varios animales (Osawa *&* Sasaki, 2004). *S. gallolyticus* es una bacteria comensal del intestino humano (Rusniok *et al*., 2010) que a diferencia de *L. plantarum*, se ha descrito que en el TGI se encuentra mayoritariamente en intestino grueso (Sekirov *et al*., 2010). Además, *S. gallolyticus* se encuentra formando parte de la microbiota del rumen de mamíferos herbívoros rumiantes. *S. gallolyticus* presenta actividad tanasa y es capaz de descarboxilar el ácido gálico hasta pirogalol (Osawa *et al*., 2000) por lo que también posee actividad galato descarboxilasa.

A pesar de que se ha descrito que *S. gallolyticus* es una bacteria capaz de degradar galotaninos, las enzimas implicadas en esta degradación permanecían desconocidas tanto genética como bioquímicamente. La comparación de las secuencias de proteínas presentes en las bases de datos con $TanA_{Sl}$ reveló la existencia de dos proteínas de *S. gallolyticus* que presentaban una identidad del 49% ($TanA_{Sg}$) y 29% ($TanB_{Sg}$) con $TanA_{Sl}$. Las proteínas $TanA_{Sg}$ y $TanB_{Sg}$ presentaban un tamaño molecular de 67 y 53 kDa y un punto isoeléctrico de 3,92 y 5,09, respectivamente. Al igual que en *L. plantarum*, se decidió conocer si todas las cepas de *S. gallolyticus* poseían ambos genes. Se analizaron *in silico* cuatro cepas de *S. gallolyticus* subsp. *gallolyticus* cuyo genoma estaba secuenciado en su totalidad. Se pudo comprobar que las cuatro cepas poseían los genes $tanA_{Sg}$ y $tanB_{Sg}$. Mediante PCR se comprobó la ausencia de ambos genes en las cepas tipo de las subespecies *S. gallolyticus* subsp. *macedonicus* y *S. gallolyticus* subsp. *pasteurianus*. Los genes que codifican enzimas tanasas no estuvieron presentes en estas subespecies por lo que se concluyó que únicamente las cepas pertenecientes a *S. gallolyticus* subsp. *gallolyticus* poseen genes que codifican proteínas con posible actividad tanasa. Al igual que en *L. plantarum*, se realizaron experimentos para conocer

la expresión de ambos genes en respuesta a la presencia de un galotanino sencillo (galato de metilo). Ambos genes presentaron un comportamiento diferente puesto que la expresión del gen *tanA*$_{Sg}$ se mantuvo invariable, sin embargo, aumentó la expresión del gen *tanB*$_{Sg}$ en respuesta a la presencia del galotanino.

Para conocer si las proteínas tanasa de *S. gallolyticus* (TanA$_{Sg}$ y TanB$_{Sg}$) presentan diferentes características bioquímicas, los genes *tanA*$_{Sg}$ (GALLO_0933) y *tanB*$_{Sg}$ (GALLO_1609) que codifican ambas proteínas en la cepa *S. gallolyticus* subsp. *gallolyticus* UCN34 se clonaron en un vector de expresión. Las proteínas tanasas recombinantes de *S. gallolyticus* se hiperprodujeron y caracterizaron bioquímicamente. Utilizando galato de metilo como substrato, la actividad específica de TanA$_{Sg}$ fue de 256 U/mg, 55% menor que la actividad específica presentada por TanB$_{Sg}$ (577 U/mg). Respecto a la temperatura y pH óptimos para su actividad, ambas proteínas presentaron un pH óptimo similar, siendo muy activas a un pH entre 6 y 8 (Figura 3 A, capítulo 4 y figura 8 A, capítulo 5). Sin embargo, ambas proteínas presentaron diferente temperatura óptima, 37 ºC para TanA$_{Sg}$ (Figura 8 B, capítulo 5) y 50-70 ºC para TanB$_{Sg}$ (Figura 3 B, capítulo 4). A pesar de que la temperatura óptima de TanA$_{Sg}$ es menor que la temperatura de TanB$_{Sg}$, TanA$_{Sg}$ es más termorresistente puesto que conservó más del 70% de su actividad máxima después de una incubación a 37 ºC durante 18 h (Figura 8 B, capítulo 5). Ambas proteínas no presentaron diferencias en el rango se substratos que utilizaron, siendo capaces de hidrolizar ésteres de los ácidos gálico y protocatéquico.

Al igual que *L. plantarum*, *S. gallolyticus* subsp. *gallolyticus* degrada galotaninos para originar pirogalol mediante una ruta que implica la acción secuencial de dos actividades enzimáticas, tanasa y galato descarboxilasa. Puesto que en esta tesis se ha caracterizado la enzima galato descarboxilasa de *L. plantarum*, se decidió buscar en el genoma de *S. gallolyticus* subsp. *gallolyticus* UCN34 proteínas similares a las proteínas LpdB, LpdC y LpdD de *L. plantarum* que codifican la enzima galato descaboxilasa. La búsqueda reveló que las proteínas codificadas por los locus GALLO_1612, GALLO_1613 y GALLO_1611 presentaron una identidad del 66, 78, y 42% con las proteínas LpdB, LpdC y LpdD de *L. plantarum*, respectivamente (Figuras 12 A, B y C). Además de la alta identidad que presentaron estas proteínas con las subunidades de la galato descarboxilasa de *L. plantarum*, se comprobó que éstas se encuentran situadas en el cromosoma de *S. gallolyticus* UCN34 muy próximas al locus que codifica TanB$_{Sg}$ (GALLO_1609). Por ello, aunque no se ha demostró experimentalmente su actividad bioquímica, los datos obtenidos apoyan que las subunidades de la enzima galato

descarboxilasa (SgdB, SgdC y SgdD) en *S. gallolyticus* están codificadas por los locus descritos anteriormente los cuales están situados en el genoma muy próximos a $TanB_{Sg}$.

A.

Subunidad B

```
SgdB   MSKKRIVVAISGASGTIYAINLLKKLKEYPDIETHVVMSDWAHENLKLELDMSHDEFASL 60
LpdB   --MKRIVVGITGASGTIYAVDLLEKLHQRPDVEVHLVMSAWAKKNLELETDYSLAQLTAL 58
          *****.*:********::**:**:: **:*.*:*** **::**:** * *  ::::*

SgdB   CDVLYSNKDLGAKIASGSFLTDGMVIVPASMKTVAGIACGFSDNLIGRAADVALKEQRKL 120
LpdB   ADATYRANDQGAAIASGSFLNDGMVIVPASMKTVAGIAYGFGDNLISRAADVTIKEQRKL 118
       .*. *  :* ** *******.***************** **.****.*****::******

SgdB   IIVPRETPLNTIHLENLTKLSRMGVQVIPPVPAFYNHPKTLQDIIDHNTAKLLDALHIRN 180
LpdB   VIVPRETPLSVIHLENLTKLAKLGAQIIPPIPAFYNHPQSIQDLVNHQTMKILDAFHIHN 178
       :********..*********:::*.*:***:*******:::**:::*:* *:***:**:*

SgdB   DYAGRWDGD 189
LpdB   ETDRRWEGD 187
       :   **:**
```

B.

Subunidad C

```
SgdC   MSEQPYDLRKVLEELKEIPGQYHETDVEIDPNAEISGVYRYIGAGGTVERPTQEGPAMTF 60
LpdC   MAEQPWDLRRVLDEIKDDPKNYHETDVEVDPNAELSGVYRYIGAGGTVQRPTQEGPAMMF 60
       *:***:***:**:*:*: * :*******:*****:*************:********* *

SgdC   NNIKGFPNVRVNIGTMASRKRVGHILHHDYKDLGHLLNKAVENPVKPVKVSKDQAPAQEV 120
LpdC   NNVKGFPDTRVLTGLMASRRRVGKMFHHDYQTLGQYLNEAVSNPVAPETVAEADAPAHDV 120
       **:****:.**  * ****:***:::****: **: **:**.*** * .*:: :***::*

SgdC   VHLATDDDFDIRKLIAAPTNTEYDAGPYITTGLVYGSTPDKSMSDVTIHRMVLEDKDTIG 180
LpdC   VYKATDEGFDIRKLVAAPTNTPQDAGPYITVGVVFGSSMDKSKSDVTIHRMVLEDKDKLG 180
       *: ***:.******:******  *******.*:*:**: *** **************.:*

SgdC   IYIMPGGRHIGAFLSEYQKLNKPMPITINIGLDPAILIGATFEPPTTPLGYNELWVAGAL 240
LpdC   IYIMPGGRHIGAFAEEYEKANKPMPITINIGLDPAITIGATFEPPTTPFGYNELGVAGAI 240
       ************ .**:* *************** ***********:***** ****:

SgdC   RNEPVQLVDSIAVDEVGIARSEFIIEGEILPNETIQEDINTHTGHAMPEFPGYNGPANPA 300
LpdC   RNQAVQLVDGVTVDEKAIARSEYTLEGYIMPNERIQEDINTHTGKAMPEFPGYDGDANPA 300
       **:.*****.::*** .*****: :** *:*** **********:********:* ****

SgdC   LNVIKVKAVTHRKDNPIMQTTIGPSEEHVSMAGIPTEASILNLVDKAIPGKVLNVYNPPA 360
LpdC   LQVIKVTAVTHRK-NAIMQSVIGPSEEHVSMAGIPTEASILQLVNRAIPGKVTNVYNPPA 359
       *:****.****** *.***:.*********************:**::****** *******

SgdC   GGGKLMTIMQIRKENPADEGIQRQAALLAFSSFKELKTVILVDEDVDIFDMNDVMWTINT 420
LpdC   GGGKLMTIMQIHKDNEADEGIQRQAALLAFSAFKELKTVILVDEDVDIFDMNDVIWTMNT 419
       ***********:*:* ***************:**********************:**:**

SgdC   RFQAHKDIMSLEGMRNHPLDPSERPEYSPEHIRVRGMSSKLVLDGTVPFDMKDQFERAKF 480
LpdC   RFQADQDLMVLSGMRNHPLDPSERPQYDPKSIRFRGMSSKLVIDGTVPFDMKDQFERAQF 479
       ****.:*:* *.*************:*.*: **.********:***************:*

SgdC   KEVPDWKKYLD 491
LpdC   MKVADWEKYLK 490
        :*.**:***.
```

C.

Subunidad D

```
SgdD   MATFEVTDYGYSIQAQVDYIGADLFIQLTGGSHPHIGTVTTYCKENADKQVVRFPSHSGR 60
LpdD   MATFTTEQAGYQMQAILQVIGYDLLIVVTGGTNPHIGDVTTLTASTVP-ETVKFPSHDGR 59
       **** . : **.:** :: ** **:* :***::**** ***   ...  :.*:****.**

SgdD   FHKDDVLADVLLEEIAELLPQNCVITSGVHVDGISKEQIAGAFEMTKELASKIGTWLKIE 120
LpdD   FHKDNFISERMAKRIQRYLAGSCTITAGIHVNQITKAQIAAAAPMTDDLSRQIISWLQAH 119
       ****:.::: : :.* . *. .*.**:*:**: *:* ***.*  **.:*: :* :**: .

SgdD   KSPISHPKYQKNTKQ-- 135
LpdD   PVQAEKPEYYGQDEQPR 136
           .:*:*  : :*
```

Figura 12. A. Comparación de las secuencias de aminoácidos de SgdB y LpdB. Los residuos idénticos se indican con (*), los residuos conservados con (:) y los semiconservados con (.). B. Comparación de las secuencias de aminoácidos de SgdC y LpdC. Los residuos idénticos se indican con (*), los residuos conservados con (:) y los semiconservados con (.).C. Comparación de las secuencias de aminoácidos de SgdD y LpdD. Los residuos idénticos se indican con (*), los residuos conservados con (:) y los semiconservados con (.).

El estudio de la degradación de galotaninos en algunas especies bacterianas presentes en el TGI humano permitió conocer las características de las proteínas tanasas responsables de este metabolismo en estas bacterias. Las características principales de estas proteínas se resumen en la Tabla 4.

Tabla 4. Características principales de las proteínas tanasas de *A. parvulum*, *L. plantarum* y *S. gallolyticus*.

Características	$TanA_{Ap}$	$TanA_{Lp}$	$TanA_{Sg}$	$TanB_{Lp}$	$TanB_{Sg}$
Tipo de proteína	Extracelular	Extracelular	Extracelular	Intracelular	Intracelular
aa /peso molecular (KDa)	607/66	626/67	596/64	469/51	470/53
Punto isoeléctrico	4.67	9.94	3.92	6.17	5.09
Peso molecular proteína madura (kDa)	64	65	61		
Actividad específica (U/mg)	3,5	39	256	408	577
mg proteína/L cultivo	12 mg/L	2,71 mg/L	4 mg/L	17 mg/L	12 mg/L
Temperatura óptima (ºC)	55	20-30	37	40	50-70
pH óptimo	6	6	6	7	7
Estabilidad (ºC)	25-45	25,30,37	25,30,37	25-37	25,30,37,45
Activadores		$CaCl_2$, DMSO, TritonX-100	Tween80, $CaCl_2$, KCl	$CaCl_2$	DMSO, $CaCl_2$,
Inhibidores	β-mercaptoetanol, $HgCl_2$, $ZnCl_2$	β-mercaptoetanol, $HgCl_2$	β-mercaptoetanol, $HgCl_2$	β-mercaptoetanol, $HgCl_2$	β-mercaptoetanol, $HgCl_2$, $ZnCl_2$
Substratos					
Galato de metilo	+	+	+	+	+
Galato de etilo	+	+	+	+	+
Galato de propilo	+	+	+	+	+
Galato de laurilo	-	-	-	+	-
Benzoato de metilo	-	-	-	-	-
Benzoato de etilo	-	-	-	-	-
4-Hidroxibenzoato de metilo	-	-	-	-	-
4-Hidroxibenzoato de etilo	-	-	-	-	-
Vanillato de metilo	-	-	-	-	-
2,4-Dihidroxibenzoato de metilo	-	-	-	-	-
3,4-Dihidroxibenzoato de etilo	+	+	+	+	+
3,5-Dihidroxibenzoato de etilo	-	-	-	-	-
Salicilato de metilo	-	-	-	-	-
Ferulato de metilo	-	-	-	-	-
Ferulato de etilo	-	-	-	-	-
Ácido elágico	-	-	-	-	-
Quercetina	-	-	-	-	-
Catequina	-	-	-	-	-
Epicatequina	-	-	-	-	-
Epigalocatequina	-	-	-	-	-
Galato de epigalocatequina	-	+	+	+	+
Ácido tánico	-	+	+	+	+

Discusión general

1. Metabolismo de galotaninos en bacterias del tracto gastrointestinal humano

Los compuestos fenólicos de la dieta son beneficiosos para la salud del consumidor. Sin embargo, se conoce poco de cuáles son sus formas bioactivas *in vivo* y de los mecanismos mediante los cuales contribuyen a prevenir enfermedades. Aunque se han realizado estudios encaminados a conocer la biodisponibilidad de los compuestos fenólicos mediante el estudio de su consumo y de la excreción de sus formas conjugadas, existen escasos estudios enfocados a conocer la aparición *in vivo* de metabolitos formados por la degradación enzimática por bacterias del colon y su posterior absorción. Los productos de hidrólisis pueden ser importantes compuestos bioactivos *in vivo* (Rechner *et al*., 2002).

La microbiota intestinal está compuesta por la totalidad de microorganismos que habitan el TGI. Las comunidades bacterianas varían en composición lo largo del tracto digestivo y se adaptan al tipo de nutrición del huésped. En términos de diversidad genética, el microbioma sobrepasa el genoma humano en 10 veces. Teniendo en cuenta estas cifras, la gran mayoría de los genes implicados en la producción de energía y en el metabolismo están presentes en el microbioma intestinal, confiriendo al huésped la posibilidad de vivir bajo dietas variadas (Moco *et al*., 2012). Hasta ahora sólo se ha identificado un pequeño número de especies bacterianas implicadas en el metabolismo de compuestos fenólicos. El metabolismo microbiano de estos compuestos no sólo es un prerrequisito para su absorción sino que también modula la actividad biológica de estos compuestos, puesto que origina la liberación de metabolitos más activos. Se ha descrito que la metabolización de los compuestos fenólicos es dieta- e individuo-dependiente (Moco *et al*., 2012). El metabolismo microbiano de los compuestos fenólicos sigue un patrón general en el cual numerosos compuestos muy diferentes estructuralmente se convierten en un número relativamente pequeño de metabolitos. Por ello en el colon se forma un reducido número de metabolitos a partir de compuestos naturales muy diversos.

Existen escasos datos en la bibliografía sobre el consumo de galotaninos. Saura-Calixto *et al*. (2007) estimaron que el consumo de compuestos fenólicos hidrolizables (entre los que se incluyen los galotaninos) en la población española es alrededor de 1250 mg/persona/día. Se conoce muy poco del destino metabólico y de la biodisponibilidad de los taninos. Para ejercer sus funciones biológicas, los taninos tienen que encontrarse disponibles para el tejido sobre el que actúan. Por ello las propiedades biológicas de los taninos dependen de su absorción en el TGI y de su biodisponibilidad. En la dieta española se ha estimado que alrededor de un 40% de

los taninos de la dieta es bioaccesible en el intestino delgado, mientras que un 46% se convierte bioaccesible en el intestino grueso. En el caso de los taninos hidrolizables se ha descrito que el sitio principal en el que se hacen bioaccesibles es en el intestino delgado (Saura-Calixto *et al*., 2007).

La figura 13 muestra un esquema de las rutas propuestas para el metabolismo de galotaninos en el TGI humano suponiendo que los metabolitos finales son ácido 3,4-metilgálico y pirogalol sulfatado.

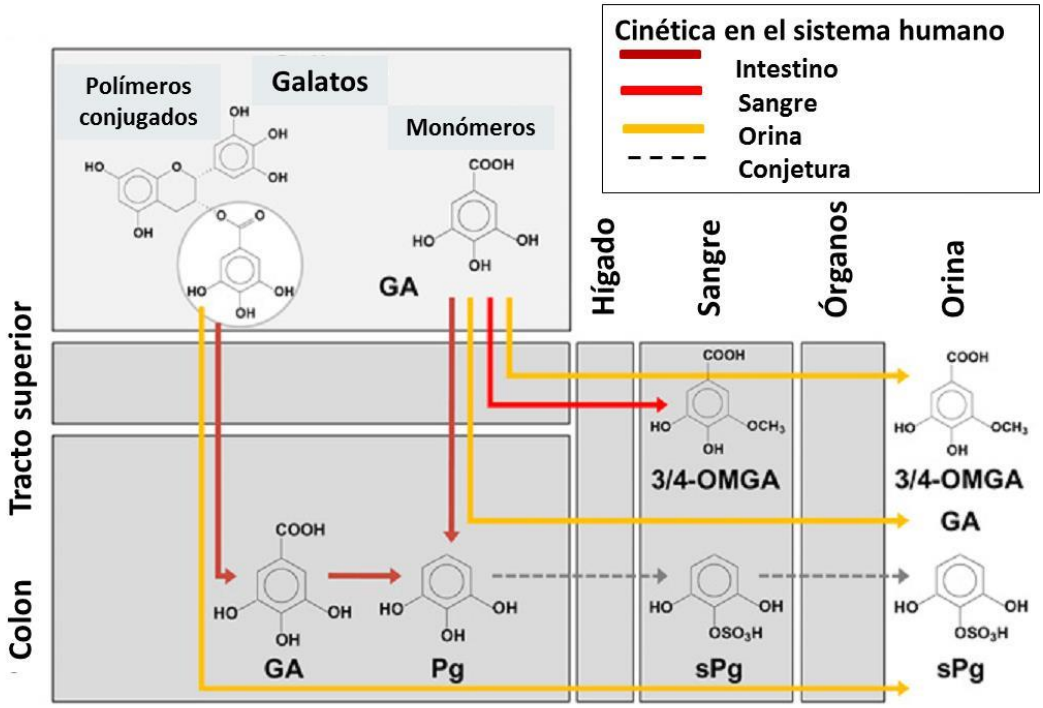

Figura 13. Rutas propuestas para el metabolismo de galotaninos y ácido gálico. Las líneas rojas, amarillas y granates muestran la información obtenida de muestras de plasma, orina (estudios in *vivo* en humanos) y modelos *in vitro*. Modificada de van Duynhoven *et al*. (2011).

La interacción de los taninos con la microbiota determina en gran medida los efectos fisiológicos de estos compuestos fenólicos. Aunque se han realizado numerosos estudios enfocados a conocer la influencia de procesado oral en las características mecánicas (por ejemplo, reológicas) y sensoriales de los alimentos, se conoce muy poco de las transformaciones que ocurren debido a la acción microbiana durante el procesado oral de los alimentos. Es interesante conocer si la hidrólisis de galotaninos en la cavidad oral puede

comenzar durante el corto periodo de tiempo del procesamiento oral. Para ello conocer la presencia de proteínas similares a tanasas entre las bacterias presentes en la cavidad oral puede aportar información interesante. Entre las bacterias presentes en la cavidad oral, las bacterias de la especie *A. parvulum* poseen un gen que codifica una proteína ($TanA_{Ap}$) similar a tanasas bacterianas descritas previamente. La producción de la enzima $TanA_{Ap}$ ha revelado que posee muy baja actividad específica posiblemente debido a que no posee residuos importantes para la actividad catalítica del enzima. La baja actividad específica que presenta $TanA_{Ap}$ puede indicar que, incluso aunque comenzase su actividad inmediatamente después de la ingestión de la comida, su contribución al metabolismo de galotaninos puede no ser relevante. A pesar de ello, las características bioquímicas que presenta la enzima son adecuadas para su acción durante el procesamiento oral de los alimentos. Durante este proceso, la saliva proporciona efectos tamponadores y se ha descrito que el pH de la saliva aumenta durante los primeros 5 minutos después de la ingesta de la comida y desciende, a pH 6 o menos, aproximadamente 15 min después (Humpfrey & Williamson, 2001). $TanA_{Ap}$ presenta actividad óptima a pH 6, por lo que en la cavidad oral puede encontrar un pH adecuado para su actividad. Respecto a la temperatura, a pesar de que la temperatura óptima para su actividad es 55 ºC, $TanA_{Ap}$ presenta un 80% de su actividad máxima a la temperatura fisiológica en humanos (37 ºC).

Por tanto, la proteína $TanA_{Ap}$ presenta características bioquímicas compatibles con su acción durante el procesamiento oral de los alimentos, puesto que la saliva le proporciona el pH y la temperatura adecuados para su actividad. El procesamiento oral ocurre durante un tiempo muy corto, sin embargo se ha descrito que durante ese corto periodo de tiempo, aproximadamente el 50% del almidón presente en el pan y el 25% del presente en la pasta se hidrolizan y transforman en moléculas más pequeñas por acción de la enzima amilasa presente en la saliva (Hoebler *et al*., 2000; Hoebler *et al*., 1998). La interacción de la enzima amilasa con el almidón produce un efecto casi inmediato en su hidrólisis, consiguiendo con ello que la comida ingerida se mezcle y digiera más fácilmente. Se podría suponer una situación similar para la acción de $TanA_{Ap}$ sobre los taninos presentes en la dieta. Sin embargo, cuando se estudió la acción de la enzima $TanA_{Ap}$ pura sobre un tanino natural complejo, como el ácido tánico, no se observó actividad hidrolítica. Existe la posibilidad de que este compuesto no sea el substrato natural de $TanA_{Ap}$, y en ese caso, el sustrato natural del enzima permanece desconocido en la actualidad. Por otro lado, no se puede excluir que durante el procesado oral de alimentos $TanA_{Ap}$ necesite para su actividad un cofactor desconocido. De los aditivos ensayados, ninguno de ellos consiguió aumentar la actividad de la proteína $TanA_{Ap}$ pura.

Los resultados indican que, aunque la actividad de la tanasa de *A. parvulum* puede comenzar casi inmediatamente después de la ingestión de comida, su contribución al metabolismo de galotaninoss puede no ser relevante. Además, resultados *in silico* indican que *A. parvulum* carece de la segunda actividad necesaria para el metabolismo de estos compuestos, la actividad galato descarboxilasa . Por ello, la mayor parte de la digestión de los taninos debe ocurrir por acción de las tanasas presentes en las bacterias intestinales.

Se ha descrito que durante la digestión en el intestino delgado, los taninos polimerizados de alto peso molecular pueden formar complejos con proteínas, almidón y enzimas digestivas, entre las que se incluyen enzimas con actividad pectinasa, amilasa, lipasa, proteasa o β-galactosidasa, dando lugar a la formación de complejos menos digeribles. Los galotaninos de menor tamaño molecular se pueden absorber más fácilmente. Respecto a la biodisponiblidad de los galotaninos en el intestino delgado, algunos estudios han evaluado su grado de hidrólisis durante la digestión enzimática en el estómago y en el intestino delgado. También se han realizado estudios para conocer el grado de absorción de los monómeros de los taninos hidrolizables. En ratas se ha comprobado que la absorción intestinal de ácido gálico es muy baja después de su administración oral (t_{max} 60 min) (Konishi *et al*., 2004).En humanos se han obtenido resultados similares, con un t_{max} de absorción de 1,27 h (Shahrzad *et al*., 2001). Se ha sugerido que pueden existir dos sistemas diferentes para la absorción gástrica del ácido gálico, un sistema de entrada rápido para el ácido gálico intacto, y un sistema de entrada lento para los derivados conjugados (Konishi *et al*., 2006). Se ha descrito que en humanos el principal metabolito de la absorción de ácido gálico es el ácido 4-*O*-metilgálico (Shahrzad *et al*., 1998).

Como se ha comentado anteriormente la microbiota no es homogénea en el TGI. La presencia de bacterias del género *Lactobacillus* es mayoritaria en el estómago e intestino delgado aunque también se han detectado en colon y heces, siendo su cantidad variable entre individuos (Kleerebezem & Vaughan, 2009). La presencia en el TGI de *L. plantarum*, especie capaz de metabolizar galotaninos, es muy importante para la absorción de estos compuestos y su influencia en la salud del consumidor. La degradación de galotaninos en *L. plantarum* implica la acción secuencial de enzimas con actividad tanasa y galato descarboxilasa, para originar pirogalol. Sin embargo, no todas las cepas de *L. plantarum* poseen la misma potencialidad metabólica para degradar taninos. Se ha descrito que la enzima tanasa $TanB_{Lp}$ está presente en todas las cepas de *L. plantarum* analizadas, sin embargo, algunas cepas de *L. plantarum* poseen además otra proteína con actividad tanasa, $TanA_{Lp}$. Esta proteína se ha

introducido en el genoma de algunas cepas de *L. plantarum* posiblemente mediante un fenómeno de transferencia horizontal del genes, a partir de un microorganismo donante desconocido. Contrariamente a la proteína tanasa de *L. plantarum* descrita previamente, $TanB_{Lp}$, la enzima $TanA_{Lp}$ parece ser una proteína extracelular puesto que cultivos de cepas de *L. plantarum* que poseen una copia funcional de $TanA_{Lp}$ son capaces de degradar galotaninos complejos presentes en el medio de cultivo.

Los estudios realizados sugieren que las dos tanasas presentes en algunas cepas de *L. plantarum* desempeñan funciones fisiológicas diferentes. Estas funciones diferentes se pueden atribuir a sus propiedades bioquímicas más que al rango de substratos que presentan. El rango de substratos es muy similar en ambas proteínas, siendo la única diferencia que $TanA_{Lp}$ no hidroliza los ésteres que tienen un sustituyente alcohol con larga cadena alifática (Curiel *et al.*, 2009). Además, en *L. plantarum* ATCC 14917^T, que posee las dos tanasas, la tanasa $TanA_{Lp}$ muestra una actividad específica 10 veces menor que la actividad específica de $TanB_{Lp}$ procedente de la misma cepa. Además, ambas proteínas presentan diferente temperatura y pH óptimos, 20-30 ºC y pH 6 para $TanA_{Lp}$ y 40 ºC y pH 7-8 para $TanB_{Lp}$ (Curiel *et al.*, 2009). A pesar de que ambas proteínas presentan alta actividad a la temperatura fisiológica humana, su actividad a pH ácido es muy reducida. Es interesante señalar que la temperatura óptima que presenta $TanA_{Lp}$ es similar a la temperatura óptima de la actividad tanasa que presentaban los extractos de la cepa *L. plantarum* ATCC 14917^T (Rodríguez *et al.*, 2008a). Estos extractos se obtuvieron a partir de cultivos crecidos en ausencia de un posible inductor, situación en la que la enzima $TanB_{Lp}$ no está inducida. Por ello se puede deducir que, en ausencia de substrato, las características bioquímicas que presentan los extractos celulares de *L. plantarum* ATCC 14917^T, que poseee las dos tanasas, son similares a las características que presenta $TanA_{Lp}$. Este resultado indica que, en ausencia de un substrato, la actividad de $TanA_{Lp}$ predomina en las cepas de *L. plantarum* que poseen dos enzimas tanasa. Los resultados de expresión génica indican que $TanA_{Lp}$ no se induce por la presencia de un éster de ácido gálico (galato de metilo); sin embargo, $tanA_{Lp}$ puede poseer un nivel basal de expresión suficiente para que se detecte su actividad en los extractos de *L. plantarum*. En un ambiente en el que los taninos complejos estén presentes, una tanasa extracelular, como $TanA_{Lp}$ puede ser la encargada de degradar parcialmente los galotaninos presentes en el exterior celular. Posteriormente, los ésteres de ácido gálico más sencillos, originados por la acción extracelular de $TanA_{Lp}$, pueden pasar al interior celular e inducir la expresión de *tanBLp* y ser degradados por $TanB_{Lp}$.

L. plantarum presenta una capacidad catabólica adaptada a la degradación de galotaninos lo que le otorga a esta especie una ventaja selectiva para vivir en ambientes en los que estos compuestos son abundantes, como es en el TGI humano. La presencia de dos tanasas en algunas cepas de *L. plantarum* le confiere una ventaja adicional para sobrevivir a los efectos adversos de los taninos de la dieta.

En *L. plantarum* el metabolismo de galotaninos implica además la descarboxilación de ácido gálico a pirogalol, por acción de una enzima con actividad galato descarboxilasa. Las descarboxilasas no-oxidativas de ácidos aromáticos están codificadas por tres genes que aparecen agrupados. Sin embargo, *L. plantarum* es la única bacteria en la que los genes que codifican estas descarboxilasas están separados en el genoma por más de 1 Mb. Esta organización inusual parece indicar una diferente organización enzimática. Sin embargo, no se conoce la actividad bioquímica de las tres subunidades diferentes. En *L. plantarum*, la proteína LpdD (Lp_0272) no parece necesaria para la actividad galato descarboxilasa, sin embargo LpdC (Lp_2945) parece ser la principal subunidad catalítica. La función de LpdB (Lp_0271) permanece desconocida. También se desconoce el mecanismo de descarboxilación seguido por este tipo de descarboxilasas.

La identificación de la enzima galato descarboxilasa implicada en la degradación de taninos completa la primera ruta de degradación de un compuesto fenólicos en una bacteria láctica. La ruta bioquímica propuesta implica que los galotaninos se hidrolizan a ácido gálico por acción de la enzima tanasa ($TanA_{Lp}$ y $TanB_{Lp}$), y el ácido gálico formado se descarboxila a pirogalol por acción de la enzima galato descarboxilasa (LpdB, LpdC y LpdD) (Figura 14). De los diferentes ésteres de ácidos fenólicos ensayados, las proteínas $TanA_{Lp}$ y $TanB_{Lp}$ puras sólo hidrolizan los ésteres de los ácidos gálico y protocatéquico, compartiendo la misma especificidad de substrato que la enzima galato descarboxilasa, que sólo descarboxila el ácido gálico y el ácido protocatéquico. Esta similitud de substrato sugiere una actividad conjunta de ambas actividades enzimáticas. Este hecho resulta más obvio cuando se analiza la localización de estos genes. Los genes que codifican la subunidad catalítica de la enzima galato descarboxilasa (LpdC o Lp_2945) y la tanasa ($TanB_{Lp}$ o Lp_2956) se encuentran separados en el genoma por sólo 6,5 kb.

En *L. plantarum* el compuesto final en la degradación de galotaninos es pirogalol. *L. plantarum* no posee las actividades enzimáticas necesarias para degradar posteriormente este compuesto. No se conoce la relevancia fisiológica en el TGI que representa la conversión de un

galotanino en pirogalol, pero es posible suponer la existencia en el TGI de otros organismos capaces de modificar posteriormente este metabolito.

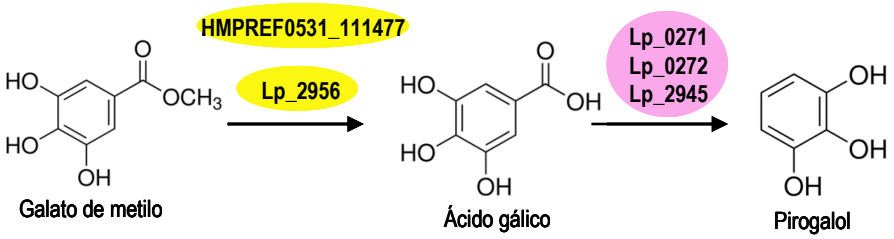

Figura 14. Representación esquemática actualizada de la degradación de galotaninos en *L. plantarum*, señalando las proteínas implicadas.

El intestino grueso es otro sitio importante del TGI en donde los taninos se convierten en biodisponibles. La mayor parte de los taninos consumidos llegan al colon, en donde, junto con otros constituyentes-no digeribles (Kahle *et al.*,2007; Gonthier *et al.*, 2003), se convierten en substratos fermentables para la microbiota bacteriana. La abundante microbiota del colon desempeña un papel crítico en el metabolismo de taninos. Tras el metabolismo microbiano de cualquier tanino que alcanza el colon, existen dos posibles rutas, la rotura de la estructura del tanino original en metabolitos absorbibles (Williamson *et al.*, 2005), o la rotura en metabolitos no absorbibles (probablemente taninos de tamaño molecular intermedio) los cuales permanecen en el lumen del colon donde pueden contrarrestar los efectos prooxidantes de la dieta.

La presencia de bacterias con actividad tanasa en las heces humanas sugiere que el ácido gálico de los galotaninos puede estar disponible durante la fermentación colónica. *S. gallolyticus* es un habitante habitual de la microbiota del colon. Al igual que en *L. plantarum*, se ha propuesto que la ruta de degradación de galotaninos implica la acción de una tanasa y una galato descarboxilasa (Chamkha *et al.* 2002), para originar pirogalol como compuesto final de la degradación (Chamkha *et al.*, 2002). El genoma de *S. gallolyticus* subsp. *gallolyticus* revela la existencia de dos proteínas con posible actividad tanasa $TanA_{Sg}$ (GALLO_1609) y $TanB_{Sg}$ (GALLO_0933). Al contrario que en *L. plantarum*, estas dos proteínas se encuentran en las 4 cepas de la especie *S. gallolyticus* subsp. *gallolyticus* analizadas, no encontrándose en cepas de

las otras subespecies. La presencia generalizada de los dos genes de tanasa en *S. gallolyticus* subsp. *gallolyticu*s contrasta con la escasa presencia del gen $TanA_{Lp}$ entre las cepas de *L. plantarum*. La organización de los genes implicados en la degradación de galotaninos es también diferente respecto a *L. plantarum*. Mientras que en *L. plantarum* los genes que codifican las distintas subunidades de la galato descarboxilasa se encuentran separados en el genoma por más de 1 Mb, sin embargo, en *S gallolyticus*, los genes que codifican la posible galato descarboxilasa están todos agrupados. En ambas especies, *L. plantarum* y *S. gallolyticus*, el gen que codifica TanB está localizado cerca de los otros genes implicados en el metabolismo de galotaninos, mientras que el gen que codifica TanA se encuentra separado en el genoma. Otra similitud entre *L. plantarum* y *S. gallolyticus* subsp. *gallolyticus* es el patrón de expresión de los genes *tanA* y *tanB*. TanB son proteínas intracelulares e inducibles por ésteres de ácido gálico (galato de metilo) mientras que TanA son proteínas extracelulares y no inducibles.

Respecto a sus características bioquímicas, aun no siendo su temperatura óptima, las enzimas $TanA_{Sg}$ y $TanB_{Sg}$ presentan adecuada actividad a la temperatura fisiológica humana (37 ºC). Respecto al pH para su actividad, $TanA_{Sg}$, la proteína extracelular, presenta un 70% de su actividad máxima a pH 4.0, mientras que $TanB_{Sg}$ a ese pH presenta menos de un 10%. Ninguna de las tanasas de *L. plantarum* presenta adecuada actividad a pH 5. Otra ventaja de las enzimas de *S. gallolyticus* respecto a las de *L. plantarum* es su mayor actividad específica. Las tanasas de *S. gallolyticus* poseen una actividad específica dos veces superior a la actividad específica de las enzimas tanasa de *L. plantarum*.

Aparte de las similitudes que presentan ambas especies de bacterias lácticas, las cepas de *S. gallolyticus* subsp. *gallolyticus* presentan claras ventajas respecto a *L. plantarum* en relación a su capacidad para metabolizar galotaninos. Todas las cepas de *S. gallolyticus* poseen dos enzimas con actividad tanasa, mientras sólo una pequeña proporción de cepas de *L. plantarum* poseen las dos enzimas. *S. gallolyticus* posee una enzima tanasa extracelular que es activa a pH ácido; y por último, las enzimas tanasas de *S. gallolyticus* poseen mayor actividad enzimática. Por todo ello, de las especies de bacterias presentes en el TGI con actividad tanasa, se puede concluir que las cepas de *S. gallolyticus* subsp. *gallolyticus* son las mejor adaptadas a degradar los taninos presentes en la dieta. Esta alta capacidad degradadora de taninos explica su presencia generalizada en el TGI de animales que consumen dietas ricas en taninos, como por ejemplo, en rumiantes, a los que la presencia de *S. gallolyticus* les confiere una ventaja selectiva.

2. Enzimas tanasas bacterianas con potencial aplicación biotecnológica.

Las enzimas tanasa son objeto de numerosos estudios debido a su importancia comercial (Chávez-González *et al.*, 2012). Las enzimas tanasas son capaces de hidrolizar taninos complejos que son los compuestos antimicrobianos naturales más importantes de origen vegetal. Estas enzimas catalizan la hidrólisis de los enlaces éster presentes en los galotaninos, taninos complejos y ésteres del ácido gálico. Las aplicaciones industriales de la enzima tanasa son múltiples, en tecnología de alimentos se utiliza para el procesado de alimentos y bebidas (Chávez-González *et al.*, 2012; Aguilar *et al.*, 2007). En la actualidad, la enzima tanasa utilizada comercialmente a nivel industrial es de origen fúngico. Los hongos producen la enzima tanasa mayoritariamente de forma extracelular. Sin embargo, la utilización comercial de estas enzimas está limitada por un conocimiento insuficiente de su inducción así como por su producción a gran escala (Aguilar *et al.*, 2007). Durante los últimos 20 años se ha realizado un gran avance en la mejora de los procesos de producción, entre los que se incluye el aislamiento de nuevas cepas productoras de tanasas, el uso de diferentes sistemas de fermentación, y métodos de purificación más baratos. La tendencia actual en este campo implica la utilización de técnicas moleculares para aumentar la producción y reducir los costes de producción. Actualmente, las enzimas tanasas disponibles comercialmente son preparaciones de tanasas fúngicas con diferentes grados de pureza y con distintas unidades de actividad catalítica en función del producto adquirido (Chávez-González *et al.*, 2013).

La diversidad de aplicaciones y condiciones en las que la enzima tanasa debe actuar hace necesario disponer de un elevado número de enzimas diferentes capaces de actuar en diferentes condiciones físico-químicas. La exploración de la diversidad microbiana puede ayudar a encontrar nuevas enzimas tanasas con propiedades interesantes (Aguilar *et al.*, 2007; Chávez-González *et al.*, 2012). Un estudio realizado por Banerjee *et al.* (2012) reveló que en las bases de datos del NCBI existían 149 secuencias de bacterias y 36 de hongos que codificaban proteínas anotadas como posibles tanasas. Puesto que algunas de ellas pertenecían a distintas cepas de la misma especie, al final redujeron el número a 77 proteínas procedentes de bacterias y 31 de hongos (Banerjee *et al.*, 2012). En esta tesis doctoral se realizó una búsqueda de proteínas pero no utilizando como criterio de búsqueda que estuviesen anotadas como "posibles tanasas" si no que presentasen identidad con proteínas tanasas bacterianas descritas previamente. Esta búsqueda reveló que el número de bacterias que poseen proteínas similares a tanasas bacterianas no es muy elevado, y sólo un reducido número pertenecen a bacterias presentes en el TGI humano, como *A. parvulum, L. plantarum* y *S. gallolyticus*. En el estudio

realizado por Banerjee *et al.* (2012) se llevaron a cabo alineamientos múltiples de las posibles tanasas bacterianas y fúngicas que revelaron la presencia en ellas de regiones conservadas. Un árbol filogenético mostró la existencia de dos grupos diferentes, uno de ellos agrupa sólo a tanasas bacterianas y el otro agrupa a tanasas bacterianas y fúngicas. Las tanasas $TanA_{Lp}$, $TanA_{Sg}$ y $TanB_{Sg}$ descritas en esta tesis aparecen en el primer grupo. Por su parte, la secuencia de la tanasa $TanA_{Ap}$ no se incluyó en el estudio realizado por Banerjee *et al.* (2012). Un alineamiento múltiple de las proteínas bacterianas descritas en esta tesis reveló que claramente se podían distinguir dos grupos de enzimas. Por un lado, las tanasas TanA ($TanA_{Sl}$, $TanA_{Lp}$, $TanA_{Sg}$ y $TanA_{Ap}$) y por otro las tanasas TanB ($TanB_{Lp}$ y $TanB_{Sg}$). Las proteínas TanA son proteínas extracelulares con un tamaño molecular de 66 kDa aproximadamente; por el contrario, las proteínas TanB son proteínas intracelulares y con un tamaño molecular aproximado de 50 kDa (Tabla 3). El grado de identidad entre ambos grupos de proteínas es menor de un 30%, aunque la identidad que presentan entre si las proteínas de un mismo grupo es similar.

Recientemente se ha descrito la estructura tridimensional de la proteína $TanB_{Lp}$ (Ren *et al.*, 2013). La proteína $TanB_{Lp}$ presenta el motivo conservado Gly_{161}-X-Ser_{163}-X-Gly_{165} típico de las serin hidrolasas, además se ha identificado la triada catalítica (Ser_{163}, His_{451} y Asp_{419}) así como los residuos encargados de establecer los contactos con los tres grupos hidroxilo del ácido gálico (Asp_{421}, Lys_{343} y Glu_{357}) (Ren *et al.*, 2013). A pesar del bajo grado de identidad encontrado entre las proteínas tanasas de los grupos TanA y TanB, una observación detallada revela que los residuos descritos en $TanB_{Lp}$ e importantes para la actividad, están generalmente conservados en ambos grupos de proteínas (Figura 11). Destacar que en las proteínas TanA, a excepción de $TanA_{Ap}$, el residuo de la triada catalítica Asp_{419} está reemplazado por un residuo de Gln. Este cambio de aminoácido fue también observado por Ren *et al.* (2013) y sugirió que el residuo conservado Asp_{421} puede realizar el papel que desempeña $Asp_{419,}$ como residuo ácido en la triada catalítica del enzima. El alineamiento de las proteínas TanA y TanB también revela que en la proteína $TanA_{Ap}$ no están conservados muchos de los residuos que se ha descrito que son importantes para su actividad, como los residuos equivalentes a Lys_{343} y Glu_{357}, implicados en los contactos con los grupos hidroxilo o, más importante, dos de los residuos de la triada catalítica (Ser_{163} y Asp_{419}). La ausencia de estos residuos también se ha observado en proteínas de otras cepas y especies de *Atopobium* (datos no mostrados). Estos importantes cambios pueden justificar la baja actividad presentada por la enzima $TanA_{Ap}$. Por otro lado, la existencia de cambios menos relevantes en la secuencia de

estas proteínas puede ser responsable de las diferencias en la actividad específica que presentan. Entre las proteínas estudiadas se comprueba que las proteínas TanB presentan mayor actividad específica que las proteínas TanA. También es interesante señalar que las proteínas de *S. gallolyticus* poseen mayor actividad que las de *L. plantarum*. La proteína $TanB_{Sg}$ es la que presenta la mayor actividad específica, siendo ésta 1,4 veces superior a la de $TanB_{Lp}$, 2,2 veces a $TanA_{Sg}$, 15 veces a $TanA_{Lp}$ y 165 veces superior a la actividad específica de $TanA_{Ap}$.

Aunque se han descrito y caracterizado diversas tanasas fúngicas, de manera general presentan características bioquímicas similares, como pH óptimo entre 5.0-6.0 y temperatura óptima alrededor de 30-40 ºC (Belmares *et al.*, 2004). Las proteínas tanasas descritas en estas tesis han mostrado un variado comportamiento en sus características bioquímicas. Respecto al pH óptimo, las tanasas TanA ($TanA_{Lp}$, $TanA_{Sg}$ y $TanA_{Ap}$) presentaron un pH óptimo para su actividad a 6.0; mientras que el pH óptimo para las proteínas TanB fue ligeramente más alto (pH de 7 para $TanB_{Lp}$ y entre 6-8 para $TanB_{Sg}$). Además se observó mayor variación en la temperatura óptima de estas enzimas. A excepción de $TanA_{Ap}$ cuya temperatura óptima es muy alta (55 ºC), las tanasas TanA presentan una temperatura óptima menor que la de las proteínas TanB, siendo las temperaturas óptimas 25-30 ºC ($TanA_{Lp}$), 37 ºC ($TanA_{Sg}$), 40 ºC ($TanB_{Lp}$) y 45 ºC ($TanB_{Sg}$). Respecto a la termoestabilidad, todas las proteínas analizadas fueron estables durante incubaciones prolongadas hasta 37 ºC. $TanA_{Ap}$ presentó también termoestabilidad en incubaciones a 45 ºC.

Se ha descrito que, de manera general, los cationes Mg^{2+} y Hg^{+} incrementan la actividad de las tanasas fúngicas, mientras que la adición de Zn^{2+} y Hg^{2+}, entre otros iones, inhibe por completo su actividad (Kar *et al.*, 2003). En el estudio realizado en esta tesis a tanasas bacterianas, se comprueba un efecto variable de los aditivos ensayados. De manera general se observa que el $CaCl_2$ aumentó la actividad enzimática y que, al igual que ocurre en las tanasas fúngicas, la presencia de Hg^{2+} y Zn^{2+} inhibe la actividad de las tanasas ensayadas. El Hg^{2+} es el estado de oxidación más frecuente del mercurio, la adición de Hg^{2+} puede provocar la degradación de los enlaces disulfuro de las cisteínas afectando por tanto a la estructura de la proteína (Torchinsky, 1981). De manera similar, el β-mercaptoetanol, que también rompe los puentes disulfuro, inhibe la actividad de las tanasas fúngicas y bacterianas.

Respecto al rango de substratos, las tanasas bacterianas analizadas sólo son capaces de hidrolizar los enlaces éster de los ácidos gálico y protocatéquico. Lo que indica que otros ácidos cinámicos sin la presencia de un grupo hidroxilo o con un sustituyente distinto a –H o –

OH en la posición 2, no son hidrolizados por las tanasas. Todas las tanasas analizadas en esta tesis ($TanA_{Sg}$, $TanA_{Ap}$, $TanA_{Lp}$ y $TanB_{Sg}$) hidrolizaron los mismos ésteres, sin embargo la tanasa bacteriana caracterizada previamente, $TanB_{Lp}$ fue la única tanasa capaz de hidrolizar el galato de laurilo, un éster con un grupo alifático más grande. A pesar de que las tanasas bacterianas descritas comparten semejanzas estructurales, las diferencias observadas son las responsables de problemas estéricos para la hidrólisis de galato de laurilo.

De los ésteres más complejos presentes en los substratos vegetales naturales ensayados, todas las enzimas tanasas fueron capaces de hidrolizar el enlace éster y liberar ácido gálico a partir de galato de epicatequina y de ácido tánico.

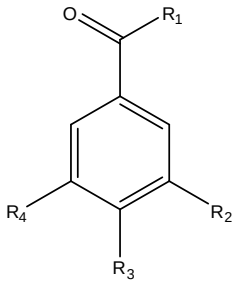

Figura 15. Representación esquemática de los sustratos potenciales de las tanasas bacterianas estudiadas. El substituyente R1 puede ser –OCH_3, -OCH_2-CH_3, --OCH_2-CH_2-CH_3, glucosa, ácido gálico o epicatequina galato.

A excepción de que algunas tanasas fúngicas presentan también actividad β-glucosidasa (Ramírez-Coronel *et al.*, 2003), las tanasas bacterianas descritas en estas tesis hidrolizan los mismos substratos que las tanasas fúngicas. A pesar de que las tanasas bacterianas analizadas no presentan similitud en su secuencia con las tanasas fúngicas, el espectro de actividad descrito es similar para ambos grupos de proteínas.

Las tanasas bacterianas pueden ser una alternativa a las tanasas fúngicas utilizadas actualmente por la industria. Puesto que cada tanasa presenta características bioquímicas diferentes, en cada aplicación o substrato puede ser recomendable el uso de una tanasa u otra. Por ello, es recomendable disponer de una tanasa para cada necesidad específica. Las tanasas bacterianas descritas en esta tesis ofrecen una amplia diversidad de características bioquímicas que pueden resultar muy útiles para su posible uso industrial.

Conclusiones

1. En las bases de datos existen proteínas bacterianas anotadas con posible actividad tanasa. Estas incluyen proteínas procedentes de especies presentes en el TGI humano como *Atopobium parvulum*, *Lactobacillus plantarum* y *Streptococcus gallolyticus* subsp. *gallolyticus*.

2. Durante el procesado oral de galotaninos no es probable que la tanasa extracelular de *A. parvulum* presente en la cavidad oral hidrolice taninos complejos debido a la baja actividad que presenta

3. Las cepas de *L. plantarum* presentes en el intestino delgado pueden metabolizar galotaninos convirtiendoles en pirogalol por la acción sucesiva de las enzimas tanasa y galato descarboxilasa. Todas las cepas de *L. plantarum* poseen una enzima tanasa intracelular inducible por ésteres de ácido gálico, mientras que sólo un reducido número de cepas de *L. plantarum* posee además una tanasa extracelular no inducible.

4. La enzima galato descarboxilasa de *L. plantarum* es una descarboxilasa no-oxidativa en la que la subunidad C es la subunidad catalítica y aunque se ha demostrado que la subunidad B es necesaria, su función permanece todavía desconocida.

5. En el intestino grueso humano se localizan cepas de *S. gallolyticus* subsp. *gallolyticus*. Estas bacterias también convierten galotaninos en pirogalol. La presencia de una tanasa extracelular y una tanasa intracelular inducible es una característica general de las cepas de *S. gallolyticus* subsp. *gallolyticus* analizadas.

6. En las condiciones ensayadas las enzimas tanasas de *S. galolyticus* subsp. *gallolyticus* poseen mayor actividad que las correspondientes enzimas de *L. plantarum* lo que parece indicar que el intestino grueso es la zona más adecuada del TGI para la hidrólisis de galotaninos.

7. Las enzimas tanasas bacterianas descritas comparten la misma especificidad de substrato sin embargo poseen características bioquímicas específicas que las hace interesantes para su posible uso industrial.

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